

## AGONIST ANTIBODIES

### TECHNICAL FIELD

This invention relates to modified antibodies containing two or more H chain V regions and two or more L chain V regions of a monoclonal antibody which show agonist activity by crosslinking a cell surface molecule(s). The modified antibodies have agonist activity of transducing a signal into cells by crosslinking a cell surface molecule(s) which can transduce a signal into cells and useful as a medicine for various purposes.

### BACKGROUND ART

JP-A 9-295999 discloses the preparation of a specific monoclonal antibody using a splenic stromal cell line as a sensitizing antigen aiming at developing specific antibodies that can recognize the aforementioned splenic stromal cells and the preparation of novel monoclonal antibodies that recognize mouse Integrin Associated Protein (mouse IAP) as an antigen. JP-A. 9-295999 also discloses that the monoclonal antibodies are capable of inducing apoptosis of myeloid cells.

WO99/12973 discloses monoclonal antibodies whose antigen is human Integrin Associated Protein (hereinafter referred to as human IAP; amino acid sequence and nucleotide sequence thereof are described in J. Cell Biol., 123, 485-496, 1993; see also Journal of Cell Science, 108, 3419-3425,

1995) and which are capable of inducing apoptosis of human nucleated blood cells (myeloid cell and lymphocyte) having said human IAP. These monoclonal antibodies are referred to antibody MABL-1 and antibody MABL-2, and hybridomas producing these antibodies are also referred to MABL-1 (FERM BP-6100) and MABL-2 (FERM BP-6101), respectively.

5 Japanese Patent Application 11-63557 describes the preparation of single chain Fvs having single chain Fv regions from the monoclonal antibodies whose antigen is 10 human IAP. The single chain Fvs are capable of inducing apoptosis of nucleated blood cells having human IAP.

15 The monoclonal antibody recognizing IAP as an antigen induces apoptosis of nucleated blood cells having human IAP, but it also causes hemagglutination in vitro. It indicates that the administration of a large amount of the monoclonal antibody recognizing IAP as an antigen may result in a side effect such as hemagglutination.

20 The inventors made intensive research for utilizing the monoclonal antibodies against human IAP as therapeutic agent of blood diseases and obtained single chain Fvs having the single chain Fv region capable of inducing apoptosis of nucleated blood cells having human IAP.

25 On the other hand modified antibodies, especially antibodies with lowered molecular size, for example, single chain Fvs were developed to improve permeability into tissues and tumors by lowering molecular size and to produce by a recombinant method. Recently the dimers of single chain

Fvs, especially hetero-dimers are used for crosslinking cells. They are bispecific modified antibodies, whose typical example is hetero-dimers of single chain Fvs recognizing antigens of cancer cells and antigens of host 5 cells like NK cells and neutrophils (Kipriyanov et al., Int. J. Cancer, 77, 9763-9772, 1998). They were produced by construction technique of single chain Fv as modified antibodies, which are more effective in treating cancers by inducing intercellular crosslinking. It has been thought 10 that the intercellular crosslinking is induced by antibodies and their fragments (e.g. Fab fragment), bispecific modified antibodies and even dimers of single chain Fvs, which are monospecific.

As antibodies capable of transducing a signal by 15 crosslinking a cell surface molecule(s), there are known an antibody against EPO receptor involved in cell differentiation and proliferation (JP-A 2000-95800), an antibody against MuSK receptor (Xie et al., Nature Biotech. 15, 768-771, 1997) and others. However there have been no 20 reports on modified antibodies with lowered molecular size.

Noticing that antibody MABL-1, antibody MABL-2 and dimers derived from them induced apoptosis of cells having IAP, the inventors discovered that they crosslink (dimerize) IAP receptor on cell surface, thereby a signal is transduced 25 into the cells and, as a result, apoptosis is induced. This suggests that monospecific single chain Fv dimers crosslink

a cell surface molecule(s) (e.g. receptor) and transduce a signal like a ligand, thereby serving as an agonist. Focusing on the intercellular crosslinking, it was discovered that the above-mentioned single chain Fv dimers 5 do not cause hemagglutination while the above-mentioned monoclonal antibodies do. The same result was also observed with single chain bivalent antibodies (single chain polypeptides containing two H chain V regions and two L chain V regions). This suggests that monoclonal antibodies 10 may form intercellular crosslinking while modified antibodies like single chain Fv dimers and single chain bivalent antibodies crosslink a cell surface molecule(s) but do not form intercellular crosslinking.

Discovering that an antibody molecule (whole IgG) 15 can be modified into single chain Fv dimers, single chain bivalent antibodies and the like which crosslink a cell surface molecule(s), thereby reducing side effects caused by intercellular crosslinking and providing new medicines inducing only desired effect on the cell, the inventors 20 completed the invention. The modified antibodies have remarkably high activity compared with original monoclonal antibodies and improved permeability into tissues due to the characteristics of having lower molecular size compared with the original antibodies and of having no constant regions.

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**DISCLOSURE OF INVENTION**

An object of this invention is to provide low molecular-size agonist modified antibodies which contain two or more H chain V regions and two or more L chain V regions of a monoclonal antibody and which combine with a cell 5 surface molecule(s) and transduce a signal into cells, thereby can serve as an agonist.

Therefore, this invention relates the modified antibodies which include two or more H chain V regions and two or more L chain V regions, preferably 2 to 4 each, 10 especially preferably two each, and show an agonist activity by crosslinking a cell surface molecule(s).

Preferred examples of the modified antibodies of the invention are dimers of the single chain Fv which contains one H chain V region and one L chain V region, or a single 15 chain polypeptide containing two H chain V regions and two L chain V regions. The H chain V region and L chain V region are preferably connected through a linker in the modified antibodies.

The above-mentioned single chain Fv dimer includes a 20 dimer by non-covalent bond, a dimer by a covalent bond through a crosslinking radical and a dimer through a crosslinking reagent (an antibody, an antibody fragment, or bivalent modified antibody). Conventional crosslinking radicals used for crosslinking peptides can be used as the 25 crosslinking radicals to form the dimers. Examples are disulfide crosslinking by cysteine residue, other crosslinking radicals such as C<sub>4</sub> - C<sub>10</sub> alkylene (e.g.

tetramethylene, pentamethylene, hexamethylene, heptamethylene and octamethylene, etc.) or C<sub>4</sub> - C<sub>10</sub> alkenylene (cis/trans -3-butenylene, cis/trans-2-pentenylene, cis/trans-3-pentenylene, cis/trans-3-hexenylene, etc.).

5 Moreover, the crosslinking reagent which can combine with a single chain Fv is, for example, an amino acid sequence which can optionally be introduced into Fv, for example, an antibody against FLAG sequence and the like or a fragment thereof, or a modified antibody originated from the 10 antibody, for example, single chain Fv.

The invention also relates to a method of inducing an agonist action to cells by administering the first ligand and the second ligand which combine with a cell surface molecule(s), and administering a substance which combine 15 with the first and the second ligands and crosslink the first and second ligands. The first ligand and the second ligand can be any things which can induce an agonist action by being crosslinked. Preferable examples are monovalent modified antibodies, such as the same or different single 20 chain Fv monomer, a fragment of antibody etc. The substance to crosslink the above-mentioned ligand can be any things that induce an agonist action to the cells by crosslinking the first ligand and the second ligand. Preferable examples are antibodies, fragments of antibodies, (Fab)<sub>2</sub> or bivalent 25 modified antibodies. Examples of bivalent antibodies are (Fab)<sub>2</sub>, dimers of single chain Fv containing one H chain V region and one L chain V region and single chain

polypeptides containing two H chain V regions and two L chain V regions. The method is effective for exploring receptors that transduce a signal into cells by crosslinking, is expected to be employed for DDS to deliver a medicine to 5 target cells and is also useful as a drug administration system which suppresses side effect and allows a medicine to become effective at desired time and for desired period.

The modified antibodies of this invention can be any things which contain L chain V region and H chain V region 10 of monoclonal antibody (e.g. antibody MABL- 1, antibody MABL-2) and which specifically recognize the cell surface molecule(s), for example, a protein (a receptor or a protein involved in signal transduction), or a sugar chain of the above-mentioned protein or of a cell membrane protein and 15 crosslink said cell surface molecule(s), thereby transduce a signal into cells. Modified antibodies in which a part of amino acid sequence of V region has been altered are included.

The present invention also relates to the 20 humanization of the above-mentioned modified antibodies. The humanized modified antibodies comprise a humanized H chain V region and/or a humanized L chain V region. Specifically, the humanized modified antibodies consist of the humanized L chain V region which comprises a framework region (FR) 25 derived from an L chain V region of human monoclonal antibody and an CDR derived from an L chain V region of mouse monoclonal antibody and/or the humanized H chain V

region which comprises an FR derived from an H chain V region of human monoclonal antibody and a CDR derived from an H chain V region of mouse monoclonal antibody. In this case, the amino acid sequence of FR or CDR may be partially altered, e.g. deleted, replaced or added.

Furthermore, the present invention relates to polypeptides which comprise an L chain C region of human antibody and an L chain V region of the mouse monoclonal antibody, and/or an H chain C region of human antibody and an H chain V region of the mouse monoclonal antibody.

The present invention also relates to modified antibodies transducing a signal into cells by combining with cell surface molecule, thereby serving as an agonist, which comprise a CDR derived from a monoclonal antibody of other mammals than mouse (such as human, rat, bovine, sheep, ape and the like), which is equivalent to said mouse CDR, or an H chain V region and an L chain V region containing the CDR. Such CDRs, H chain V regions and L chain V regions may include CDRs derived from a human monoclonal antibody prepared from, for example, a transgenic mouse or the like, and H chain V regions and L chain V regions derived from a human monoclonal antibody containing the CDR.

The invention also relates to DNAs encoding the various modified antibodies as mentioned above and genetic engineering techniques for the producing recombinant vectors comprising the DNAs.

The invention also relates to host cells transformed with the recombinant vectors. Examples of host cells are animal cells such as human cells, mouse cells or the like and microorganisms such as E. coli, Bacillus subtilis, yeast or the like.

The invention relates to a process for producing the modified antibodies, which comprises culturing the above-mentioned hosts and extracting the modified antibodies from the culture thereof.

The present invention further relates to a process for producing a dimer of the single chain Fv which comprises culturing host animal cells producing the single chain Fv in a serum-free medium to secrete the single chain Fv into the medium and isolating the dimer of the single chain Fv formed in the medium.

The present invention also relates to the use of the modified antibodies as an agonist. That is, it relates to the signal-transduction agonist which comprises as an active ingredient the modified antibody obtained as mentioned above. Since the modified antibodies used in the invention are those that crosslink the receptor on the cell surface and induce signal transduction, the receptor can be any receptor that is oligomerized, e.g. dimerized, by combining with the ligand and thereby transduce a signal into cells. The receptor includes hormone receptors and cytokine receptors. The hormone receptor includes, for example, estrogen receptor. The cytokine receptor and the

like include hematopoietic factor receptor, lymphokine receptor, growth factor receptor, differentiation control factor receptor and the like. Examples of cytokine receptors are erythropoietin (EPO) receptor, thrombopoietin (TPO) receptor, granulocyte colony stimulating factor (G-CSF) receptor, macrophage colony stimulating factor (M-CSF) receptor, granular macrophage colony stimulating factor (GM-CSF) receptor, tumor necrosis factor (TNF) receptor, interleukin-1 (IL-1) receptor, interleukin-2 (IL-2) receptor, interleukin-3 (IL-3) receptor, interleukin-4 (IL-4) receptor, interleukin-5 (IL-5) receptor, interleukin-6 (IL-6) receptor, interleukin-7 (IL-7) receptor, interleukin-9 (IL-9) receptor, interleukin-10 (IL-10) receptor, interleukin-11 (IL-11) receptor, interleukin-12 (IL-12) receptor, interleukin-13 (IL-13) receptor, interleukin-15 (IL-15) receptor, interferon-alpha (IFN-alpha) receptor, interferon-beta (IFN-beta) receptor, interferon-gamma (IFN-gamma) receptor, growth hormone (GH) receptor, insulin receptor, blood stem cell proliferation factor (SCF) receptor, vascular epidermal growth factor (VEGF) receptor, epidermal cell growth factor (EGF) receptor, nerve growth factor (NGF) receptor, fibroblast growth factor (FGF) receptor, platelet-derived growth factor (PDGF) receptor, transforming growth factor-beta (TGF-beta) receptor, leukocyte migration inhibitory factor (LIF) receptor, ciliary neurotrophic factor (CNTF) receptor, oncostatin M (OSM) receptor, Notch family receptor and the like.

Therefore, the pharmaceutical preparations containing the agonist modified antibody as an active ingredient are useful for as, for example, preventives and/or remedies for various disease such as cancers, inflammation, hormone disorders and 5 blood diseases.

The modified antibodies of the present invention comprise two or more H chain V regions and two or more L chain V regions derived from monoclonal antibodies. The structure of the modified antibodies may be a dimer of 10 single chain Fv comprising one H chain V region and one L chain V region or a polypeptide comprising two H chain V regions and two L chain V regions. In the modified antibodies of the invention, the V regions of H chain and L chain are preferably linked through a peptide linker which 15 consists of one or more amino acids. The resulting modified antibodies contain variable regions of the parent antibodies and retain the complementarity determining region (CDR) thereof, and therefore bind to the antigen with the same specificity as that of the parent monoclonal antibodies.

20 H chain V region

In the present invention, the H chain V region derived from a monoclonal antibody recognizes a cell surface molecule(s), for example, a protein (a receptor or a protein involved in signal transduction) or a sugar chain of the 25 protein or on cell membrane and oligomerizes, for example, dimerizes through crosslinking of said molecule, and thereby serves as an agonist transducing a signal into the cells.

The H chain V region of the invention includes H chain V regions derived from a mammal (e.g. human, mouse, rat, bovine, sheep, ape etc.) and partially modified H chain V regions thereof. More preferable is a humanized H chain V region containing FR of H chain V region of a human monoclonal antibody and CDR of H chain V region of a mouse monoclonal antibody. The H chain V region further can be an H chain V region derived from a human monoclonal antibody corresponding to the aforementioned H chain V region of mouse monoclonal antibody, which can be produced by recombination technique. The H chain V region of the invention may be a fragment of aforementioned H chain V region, which fragment preserves the antigen binding capacity.

15 L chain V region

In the present invention, the L chain V region derived from the monoclonal antibody recognizes a cell surface molecule(s), for example, a protein (a receptor or a protein involved in signal transduction) or a sugar chain of the protein or on cell membrane and oligomerizes, for example, dimerizes through crosslinking of said molecule, and thereby serves as an agonist transducing a signal into the cells. The L chain V region of the invention includes L chain V regions derived from a mammal (e.g. human, mouse, rat, bovine, sheep, ape etc.) and partially modified L chain V regions thereof. More preferable is a humanized L chain V region containing FR of L chain V region of human monoclonal

antibody and CDR of L chain V region of mouse monoclonal antibodies. The L chain V regions further can be an L chain V region derived from human monoclonal antibody corresponding to the aforementioned L chain V region of mouse monoclonal antibody, which can be produced by recombination technique. The L chain V regions of the invention may be fragments of L chain V region, which fragments preserve the antigen binding capacity.

Complementarity determining region (CDR)

10 Each V region of L chain and H chain forms an antigen-binding site. The variable region of the L and H chains is composed of comparatively conserved four common framework regions linked to three hypervariable regions or complementarity determining regions (CDR) (Kabat, E.A. et al., "Sequences of Protein of Immunological Interest", US Dept. Health and Human Services, 1983).

15 Major portions in the four framework regions (FRs) form  $\beta$ -sheet structures and thus three CDRs form a loop. CDRs may form a part of the  $\beta$ -sheet structure in certain cases. The three CDRs are held sterically close position to each other by FR, which contributes to the formation of the antigen-binding site together with three CDRs.

20 These CDRs can be identified by comparing the amino acid sequence of V region of the obtained antibody with known amino acid sequences of V regions of known antibodies according to the empirical rule in Kabat, E.A. et al., "Sequences of Protein of Immunological Interest".

Single chain Fv

A single chain Fv is a polypeptide monomer comprising an H chain V region and an L chain V region linked each other which are derived from monoclonal antibodies. The resulting single chain Fvs contain variable regions of the parent monoclonal antibodies and preserve the complementarity determining region thereof, and therefore the single chain Fvs bind to the antigen by the same specificity as that of the parent monoclonal antibodies (JP- Appl. 11-63557). A part of the variable region and/or CDR of the single chain Fv of the invention or a part of the amino acid sequence thereof may be partially altered, for example deleted, replaced or added. The H chain V region and L chain V region composing the single chain Fv of the invention are mentioned before and may be linked directly or through a linker, preferably a peptide linker. The constitution of the single chain Fv may be [H chain V region]-[L chain V region] or [L chain V region]-[H chain V region]. In the present invention, it is possible to make the single chain Fv to form a dimer, a trimer or a tetramer, from which the modified antibody of the invention can be formed.

Single chain modified antibody

The single chain modified antibodies of the present invention comprising two or more H chain V regions and two or more L chain V regions, preferably each two to four, especially preferable each two comprise two or more H chain V regions and L chain V regions as mentioned above.

Each region of the peptide should be arranged such that the modified single chain antibody forms a specific steric structure, concretely mimicking a steric structure formed by the dimer of single chain Fv. For instance, the V regions are arranged in the order of the following manner:

5 [H chain V region]-[L chain V region]-[H chain V region]-[L chain V region]; or

[L chain V region]-[H chain V region]-[L chain V region]-[H chain V region],

10 wherein these regions are connected through a peptide linker, respectively.

Linker

In this invention, the linkers for the connection between the H chain V region and the L chain V region may be 15 any peptide linker which can be introduced by the genetic engineering procedure or any linker chemically synthesized. For instance, linkers disclosed in literatures, e.g. Protein Engineering, 9(3), 299-305, 1996 may be used in the invention. These linkers can be the same or different in 20 the same molecule. If peptide linkers are required, the following are cited as example linkers:

Ser

Gly-Ser

Gly-Gly-Ser

25 Ser-Gly-Gly

Gly-Gly-Gly-Ser

Ser-Gly-Gly-Gly

Gly-Gly-Gly-Gly-Ser  
Ser-Gly-Gly-Gly-Gly  
Gly-Gly-Gly-Gly-Gly-Ser  
Ser-Gly-Gly-Gly-Gly-Gly  
5           Gly-Gly-Gly-Gly-Gly-Ser  
          Ser-Gly-Gly-Gly-Gly-Gly  
          (Gly-Gly-Gly-Gly-Ser)<sub>n</sub> and  
          (Ser-Gly-Gly-Gly-Gly)<sub>n</sub>

wherein n is an integer not less than one. Preferable  
10 length of the linker peptide varies dependent upon the  
receptor to be the antigen, in the case of single chain Fvs,  
the range of 1 to 20 amino acids is normally preferable. In  
the case of single chain modified antibodies comprising two  
or more H chain V regions and two or more L chain V regions,  
15 the peptide linkers connecting those forming the same  
antigen binding site comprising [H chain V region]-[L chain  
V region] (or [L chain V region]-[H chain V region]) have  
lengths of 1 - 30 amino acids, preferably 1 - 20 amino  
acids, more preferably 3 - 18 amino acids. The peptide  
linkers connecting those not forming the same antigen biding  
20 site comprising [H chain V region]-[L chain V region] or ([L  
chain V region]-[H chain V region]) have lengths of 1 - 40  
amino acids, preferably 3 - 30 amino acids, more preferably  
5 - 20 amino acids. The method for introducing those linkers  
25 will be described in the explanation for DNA construction  
coding for modified antibodies of the invention.

The chemically synthesized linkers, i.e. the chemical crosslinking agents, according to the invention can be any linkers conventionally employed for the linkage of peptides. Examples of the linkers may include N-hydroxy succinimide (NHS), disuccinimidyl suberate (DSS), bis(sulfosuccinimidyl)suberate (BS<sup>3</sup>), dithiobis(succinimidyl propionate) (DSP), dithiobis(sulfosuccinimidyl propionate) (DTSSP), ethylene glycolbis(succinimidyl succinate) (EGS), ethylene glycolbis(sulfosuccinimidyl succinate) (sulfo-EGS), disuccinimidyl tartrate (DST), disulfosuccinimidyl tartrate (sulfo-DST), bis[2-(succinimido oxycarbonyloxy)ethyl]sulfone (BSOCOES), bis[2-(sulfosuccinimido oxycarbonyloxy)ethyl]sulfone (sulfo-BSOCOES) or the like. These are commercially available.

To form a dimer of the single chain Fv it is preferable to select a linker suitable to dimerize in the solution such as culture medium more than 20%, preferably more than 50%, more preferably more than 80%, most preferably more than 90% of the single chain Fv produced in the host cells. Specifically, preferable is a linker composed of 2 to 12 amino acids, preferably 3 to 10 amino acids or other linkers corresponding thereto.

Preparation of modified antibodies

The modified antibodies can be produced by connecting, through the aforementioned linker, an H chain V region and an L chain V region derived from known or novel monoclonal antibodies specifically binding to a cell surface

molecule(s). As examples of the single chain Fvs are cited MABL1-scFv and MABL2-scFv comprising the H chain V region and the L chain V region derived from the antibody MABL-1 and the antibody MABL-2, respectively. As examples of the 5 single chain polypeptides comprising two H chain V regions and two L chain V regions are cited MABL1-sc(Fv)<sub>2</sub> and MABL2-sc(Fv)<sub>2</sub> comprising the H chain V region and the L chain V region derived from the aforementioned antibodies.

For the preparation of the polypeptide, a signal 10 peptide may be attached to N-terminal of the polypeptide if the polypeptide is desired to be a secretory peptide. A well-known amino acid sequence useful for the purification of polypeptide such as the FLAG sequence may be attached for the efficient purification of the polypeptide. In this case 15 a dimer can be formed by using anti-FLAG antibody.

For the preparation of the modified antibody of the invention, it is necessary to obtain a DNA, i.e. a DNA 20 encoding the single chain Fv or a DNA encoding reconstructed single chain polypeptide. These DNAs, especially for MABL1-scFv, MABL2-scFv, MABL1-sc(Fv)<sub>2</sub> and/or MABL2-sc(Fv)<sub>2</sub> are obtainable from the DNAs encoding the H chain V region and the L chain V region derived from said Fv. They are also obtainable by PCR method using those DNA as a template and amplifying the part of DNA contained therein encoding 25 desired amino acid sequence with the aid of a pair of primers corresponding to both ends thereof.

5        In the case where each V region having partially modified amino acid sequence is desired, the V regions in which one or some amino acids are modified, i.e. deleted, replaced or added can be obtained by a procedure known in the art using PCR. A part of the amino acid sequence in the V region is preferably modified by the PCR known in the art in order to prepare the modified antibody which is sufficiently active against the specific antigen.

10       For the determination of primers for the PCR amplification, it is necessary to decide the type of the H chain and L chain of the desired antibodies. In the case of antibody MABL-1 and the antibody MABL-2 it has been reported, however, that the antibody MABL-1 has  $\kappa$  type L chains and  $\gamma 1$  type H chains and the antibody MABL-2 has  $\kappa$  type L chains and  $\gamma 2a$  type H chains (JP-Appl. 11-63557). For the PCR amplification of the DNA encoding the H chain and L chain of the antibody MABL-1 and/or the antibody MABL-2, primers described in Jones, S.T. et al., Bio/Technology, 9, 88-89, 1991 may be employed.

20       For the amplification of the L chain V regions of the antibody MABL-1 and the antibody MABL-2 using the polymerase chain reaction (PCR), 5'-end and 3'-end oligonucleotide primers are decided as aforementioned. In the same manner, 5'-end and 3'-end oligonucleotide primers are decided for the amplification of the H chain V regions of the antibody MABL-1 and the antibody MABL-2.

5 In embodiments of the invention, the 5'-end primers which contain a sequence "GANTC" providing the restriction enzyme Hinf I recognition site at the neighborhood of 5'-terminal thereof are used and the 3'-end primers which contain a nucleotide sequence "CCCGGG" providing the XmaI recognition site at the neighborhood of 10 5'-terminal thereof are used. Other restriction enzyme recognition site may be used instead of these sites as long as they are used for subcloning a desired DNA fragment into a cloning vector.

15 Specifically designed PCR primers are employed to provide suitable nucleotide sequences at 5'-end and 3'-end of the cDNAs encoding the V regions of the antibodies MABL-1 and MABL-2 so that the cDNAs are readily inserted into an expression vector and appropriately function in the expression vector (e.g. this invention devises to increase transcription efficiency by inserting Kozak sequence). The V regions of the antibodies MABL-1 and MABL-2 obtained by 20 amplifying by PCR using these primers are inserted into HEF expression vector containing the desired human C region (see WO92/19759). The cloned DNAs can be sequenced by using any conventional process which comprises, for example, inserting the DNAs into a suitable vector and then sequencing using the automatic DNA sequencer (Applied Biosystems).

25 A linker such as a peptide linker can be introduced into the modified antibody of the invention in the following manner. Primers which have partially

complementary sequence with the primers for the H chain V regions and the L chain V regions as described above and which code for the N-terminal or the C-terminal of the linker are designed. Then, the PCR procedure can be carried 5 out using these primers to prepare a DNA encoding the peptide linker having desired amino acid sequence and length. The DNAs encoding the H chain V region and the L chain V region can be connected through the resulting DNA to produce the DNA encoding the modified antibody of the 10 invention which has the desired peptide linker. Once the DNA encoding one of the modified antibodies is prepared, the DNAs encoding the modified antibodies with or without the desired peptide linker can readily be produced by designing various primers for the linker and then carrying out the PCR 15 using the primers and the aforementioned DNA as a template.

Each V region of the modified antibody of the present invention can be humanized by using conventional techniques (e.g. Sato, K. et al., Cancer Res., 53, 1-6 20 (1993)). Once a DNA encoding a humanized Fv is prepared, a humanized single chain Fv, a fragment of the humanized single chain Fv, a humanized monoclonal antibody and a fragment of the humanized monoclonal antibody can readily be produced according to conventional methods. Preferably, amino acid sequences of the V regions thereof may be 25 partially modified, if necessary.

Furthermore, a DNA derived from other mammalian origin, for example a DNA of human, can be produced in the

same manner as used to produce DNA encoding the H chain V region and the L chain V region derived from mouse mentioned in the above. The resulting DNA can be used to prepare an H chain V region and an L chain V region of other mammal, especially human origin, a single chain Fv derived from human and a fragment thereof, and a monoclonal antibody of human origin and a fragment thereof.

As mentioned above, when the aimed DNAs encoding the V regions of the modified antibodies and the V regions of the humanized modified antibodies are prepared, the expression vectors containing them and hosts transformed with the vectors can be obtained according to conventional methods. Further, the hosts can be cultured according to a conventional method to produce the reconstructed single chain Fv, the reconstructed humanized single chain Fv, the humanized monoclonal antibodies and fragments thereof. They can be isolated from cells or a medium and can be purified into a homogeneous mass. For this purpose any isolation and purification methods conventionally used for proteins, e.g. chromatography, ultra-filtration, salting-out and dialysis, may be employed in combination, if necessary, without limitation thereto.

When the reconstructed single chain Fv of the present invention is produced by culturing an animal cell such as COS7 cells or CHO cells, preferably CHO cells, in a serum-free medium, the reconstructed single chain Fv is efficiently dimerized in the medium. The dimer of the single

chain Fv as formed above can be isolated stably and efficiently and preserved for a long period in the dimer form. The serum-free medium employed in the invention may be any medium conventionally used for the production of a recombinant protein without limit thereto.

For the production of the modified antibodies of the present invention, any expression systems can be employed, for example, eukaryotic cells such as animal cells, e.g., established mammalian cell lines, filamentous fungi and yeast, and prokaryotic cells such as bacterial cells e.g., E. coli. Preferably, the modified antibodies of the invention are expressed in mammalian cells, for example COS7 cells or CHO cells.

For the production of the reconstructed polypeptides binding to cells with human IAP of the present invention, any expression systems can be employed, for example, eukaryotic cells such as animal cells, e.g., established mammalian cell lines, filamentous fungi and yeast, and prokaryotic cells such as bacterial cells e.g., E. coli. Preferably, the reconstructed polypeptides of the invention are expressed in mammalian cells, for example COS7 cells or CHO cells.

In these cases, conventional promoters useful for the expression in mammalian cells can be used. Preferably, human cytomegalovirus (HCMV) immediate early promoter is used. Expression vectors containing the HCMV promoter

include HCMV-VH-HC $\gamma$  1, HCMV-VL-HCK and the like which are derived from pSV2neo (WO92/19759).

5        Additionally, other promoters for gene expression in mammal cell which may be used in the invention include virus promoters derived form retrovirus, polyoma virus, adenovirus and simian virus 40 (SV40) and promoters derived from mammal such as human polypeptide-chain elongation factor-1 $\alpha$  (HEF-1 $\alpha$ ). SV40 promoter can easily be used according to the method of Mulligan, R.C., et al. (Nature 10 277, 108-114 (1979)) and HEF-1 $\alpha$  promoter can also be used according to the methods of Mizushima, S. et al. (Nucleic Acids Research, 18, 5322 (1990)).

15        Replication origin (ori) which can be used in the invention includes ori derived from SV40, polyoma virus, adenovirus, bovine papilloma virus (BPV) and the like. An expression vector may contain, as a selection marker, phosphotransferase APH (3') II or I (neo) gene, thymidine kinase (TK) gene, E. coli xanthine-guanine phosphoribosyl transferase (Ecogpt) gene or dihydrofolate reductase (DHFR) 20 gene.

25        The antigen-binding activity of the modified antibody as prepared above can be evaluated using the binding-inhibitory ability of original antibodies as an index. Concretely, the activity is evaluated in terms of the absence or presence of concentration-dependent inhibition of the binding of said monoclonal antibody as an index.

More in detail, animal cells transformed with an expression vector containing a DNA encoding the modified antibody of the invention, e.g., COS7 cells or CHO cells, are cultured. The cultured cells and/or the supernatant of the medium or the modified antibody purified from them are used to determine the binding to antigen. As a control is used a supernatant of the culture medium in which cells transformed only with the expression vector were cultured. In the case of an antigen, for example, the antibody MABL-1 and the antibody MABL-2, a test sample of the modified antibody of the invention or the supernatant of the control is added to mouse leukemia cell line, L1210 cells, expressing human IAP and then an assay such as the flow cytometry is carried out to evaluate the antigen-binding activity.

In vitro evaluation of the signal transduction effect (apoptosis-inducing effect in the cases of the antibody MABL-1 and the antibody MABL-2) is performed in the following manner: A test sample of the above modified antibody is added to the cells which are expressing the antibody or cells into which the gene for the antibody has been introduced, and is evaluated by the change caused by the signal transduction, for example, whether cell death is induced in a manner specific to the human IAP-antigen.

In vivo evaluation of the apoptosis-inducing effect, for example, in the case where the modified antibody recognizes human IAP (e.g. modified antibodies derived from

the antibody MABL-1 and the antibody MABL-2) is carried out in the following manner: A mouse model of human myeloma is prepared. To the mice is intravenously administered the monoclonal antibody or the modified antibody of the invention, which induces apoptosis of nucleated blood cells having IAP. To mice of a control group is administered PBS alone. The induction of apoptosis is evaluated in terms of antitumor effect based on the change of human IgG content in serum of the mice and their survival time.

10 The modified antibodies of the invention, which comprises two or more H chain V regions and two or more L chain V regions, preferably each two to four, more preferably each two, may be a dimer of the single chain Fv comprising one H chain V region and one L chain V region, or 15 a single chain polypeptide in which two or more H chain V regions and two or more L chain V regions are connected. It is considered that owing to such construction the peptide mimics three dimensional structure of the antigen binding site of the parent monoclonal antibody and therefore retains 20 an excellent antigen-binding property.

25 The modified antibodies of the invention has been remarkably lowered in the molecular size compared with antibody molecule (whole IgG), and, therefore, have superior permeability into tissues and tumors and higher activity than original monoclonal antibodies. Therefore, it is possible to transduce various signals into cells by properly selecting the original antibody which is modified. The

pharmaceutical preparations containing them are useful for treating diseases curable by inducing signal transduction, for example cancers, inflammation, hormone disorders as well as blood dyscrasia, for example, leukemia, malignant 5 lymphoma, aplastic anemia, myelodysplasia syndrome and polycythemia vera. It is further expected that the antibody of the invention can be used as a contrast agent by RI-labeling. The effect can be enhanced by attaching to a RI-compound or a toxin.

10 The present invention is illustrated by examples, which by no means restrict the scope of the invention, using monoclonal antibodies binding to human IAP (the antibody MABL-1 and the antibody MABL-2).

15 **BEST MODE FOR WORKING THE INVENTION**

The present invention will concretely be illustrated in reference to the following examples, which in no way limit the scope of the invention.

20 For illustrating the production process of the modified antibodies of the invention, examples of producing single chain Fvs are shown below. Mouse antibodies against human IAP, MABL-1 and MABL-2 were used in the examples of producing the modified antibodies. Hybridomas MABL-1 and MABL-2 producing them respectively were internationally 25 deposited as FERM BP-6100 and FERM BP-6101 with the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Minister of International

Trade and Industry (1-3 Higasi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan), an authorized depository for microorganisms, on September 11, 1997.

5 Example 1 (Cloning of DNAs encoding V region of mouse monoclonal antibodies to human IAP)

DNAs encoding variable regions of the mouse monoclonal antibodies to human IAP, MABL-1 and MABL-2, were cloned as follows.

10 1.1 Preparation of messenger RNA (mRNA)

mRNAs of the hybridomas MABL-1 and MABL-2 were obtained by using mRNA Purification Kit (Pharmacia Biotech).

1.2 Synthesis of double-stranded cDNA

Double-stranded cDNA was synthesized from about 1 15  $\mu$ g of the mRNA using Marathon cDNA Amplification Kit (CLONTECH) and an adapter was linked thereto.

1.3 PCR Amplification of genes encoding variable regions of an antibody by

PCR was carried out using Thermal Cycler (PERKIN 20 ELMER).

(1) Amplification of a gene coding for L chain V region of MABL-1

Primers used for the PCR method are Adapter Primer-1 (CLONTECH) shown in SEQ ID No. 1, which hybridizes 25 to a partial sequence of the adapter, and MKC (Mouse Kappa Constant) primer (Bio/Technology, 9, 88-89, 1991) shown in

SEQ ID No. 2, which hybridizes to the mouse kappa type L chain V region.

50  $\mu$ l of the PCR solution contains 5  $\mu$ l of 10  $\times$  PCR Buffer II, 2 mM MgCl<sub>2</sub>, 0.16 mM dNTPs (dATP, dGTP, dCTP and dTTP), 2.5 units of a DNA polymerase, AmpliTaq Gold (PERKIN ELMER), 0.2  $\mu$ M of the adapter primer of SEQ ID No. 1, 0.2  $\mu$ M of the MKC primer of SEQ ID No. 2 and 0.1  $\mu$ g of the double-stranded cDNA derived from MABL-1. The solution was preheated at 94°C of the initial temperature for 9 minutes and then heated at 94°C for 1 minute, at 60°C for 1 minute and at 72°C for 1 minute 20 seconds in order. This temperature cycle was repeated 35 times and then the reaction mixture was further heated at 72°C for 10 minutes.

10 (2) Amplification of cDNA encoding H chain V region of MABL-

15 1

The Adapter Primer-1 shown in SEQ ID No. 1 and MHC- $\gamma$ 1 (Mouse Heavy Constant) primer (Bio/Technology, 9, 88-89, 1991) shown in SEQ ID No. 3 were used as primers for PCR.

20 The amplification of cDNA was performed according to the method of the amplification of the L chain V region gene, which was described in Example 1.3-(1), except for using 0.2  $\mu$ M of the MHC- $\gamma$ 1 primer instead of 0.2  $\mu$ M of the MKC primer.

25 (3) Amplification of cDNA encoding L chain V region of MABL-

2

The Adapter Primer-1 of SEQ ID No. 1 and the MKC primer of SEQ ID No. 2 were used as primers for PCR.

The amplification of cDNA was carried out according to the method of the amplification of the L chain V region gene of MABL-1 which was described in Example 1.3-5 (1), except for using 0.1  $\mu$ g of the double-stranded cDNA derived from MABL-2 instead of 0.1  $\mu$ g of the double-stranded cDNA from MABL-1.

(4) Amplification of cDNA encoding H chain V region of MABL-

10 2

The Adapter Primer-1 of SEQ ID No. 1 and MHC- $\gamma$ 2a primer (Bio/Technology, 9, 88-89, 1991) shown in SEQ ID No. 4 were used as primers for PCR.

The amplification of cDNA was performed according to the method of the amplification of the L chain V region gene, which was described in Example 1.3-(3), except for using 0.2  $\mu$ M of the MHC- $\gamma$ 2a primer instead of 0.2  $\mu$ M of the MKC primer.

1.4 Purification of PCR products

20 The DNA fragment amplified by PCR as described above was purified using the QIAquick PCR Purification Kit (QIAGEN) and dissolved in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA.

1.5 Ligation and Transformation

25 About 140 ng of the DNA fragment comprising the gene encoding the mouse kappa type L chain V region derived from MABL-1 as prepared above was ligated with 50 ng of

pGEM-T Easy vector (Promega) in the reaction buffer comprising 30 mM Tris-HCl (pH 7.8), 10 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 1 mM ATP and 3 units of T4 DNA Ligase (Promega) at 15°C for 3 hours.

5 Then, 1 µl of the reaction mixture was added to 50 µl of E. coli DH5 $\alpha$  competent cells (Toyobo Inc.) and the cells were stored on ice for 30 minutes, incubated at 42°C for 1 minute and stored on ice for 2 minutes again. 100 µl of SOC medium (GIBCO BRL) was added. The cells of E. coli were plated on LB (Molecular Cloning: A Laboratory Manual, 10 Sambrook et al., Cold Spring Harbor Laboratory Press, 1989) agar medium containing 100 µg/ml of ampicillin (SIGMA) and cultured at 37°C overnight to obtain the transformant of E. coli.

15 The transformant was cultured in 3 ml of LB medium containing 50 µg/ml of ampicillin at 37°C overnight and the plasmid DNA was prepared from the culture using the QIAprep Spin Miniprep Kit (QIAGEN).

20 The resulting plasmid comprising the gene encoding the mouse kappa type L chain V region derived from the hybridoma MABL-1 was designated as pGEM-M1L.

According to the same manner as described above, a plasmid comprising the gene encoding the mouse H chain V region derived from the hybridoma MABL-1 was prepared from 25 the purified DNA fragment and designated as pGEM-M1H.

A plasmid comprising the gene encoding the mouse kappa type L chain V region derived from the hybridoma MABL-

2 was prepared from the purified DNA fragment and designated as pGEM-M2L.

5 A plasmid comprising the gene encoding the mouse H chain V region derived from the hybridoma MABL-2 was prepared from the purified DNA fragment and designated as pGEM-M2H.

**Example 2 (DNA Sequencing)**

10 The nucleotide sequence of the cDNA encoding region in the aforementioned plasmids was determined using Auto DNA Sequencer (Applied Biosystem) and ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystem) according to the manufacturer's protocol.

15 The nucleotide sequence of the gene encoding the L chain V region from the mouse antibody MABL-1, which is included in the plasmid pGEM-M1L, is shown in SEQ ID No. 5.

20 The nucleotide sequence of the gene encoding the H chain V region from the mouse antibody MABL-1, which is included in the plasmid pGEM-M1H, is shown in SEQ ID No. 6.

The nucleotide sequence of the gene encoding the L chain V region from the mouse antibody MABL-2, which is included in the plasmid pGEM-M2L, is shown in SEQ ID No. 7.

25 The nucleotide sequence of the gene encoding the H chain V region from the mouse antibody MABL-2, which is included in the plasmid pGEM-M2H, is shown in SEQ ID No. 8.

**Example 3 (Determination of CDR)**

The V regions of L chain and H chain generally have a similarity in their structures and each four framework regions therein are linked by three hypervariable regions, i.e., complementarity determining regions (CDR). An 5 amino acid sequence of the framework is relatively well conserved, while an amino acid sequence of CDR has extremely high variation (Kabat, E.A., et al., "Sequences of Proteins of Immunological Interest", US Dept. Health and Human Services, 1983).

10 On the basis of these facts, the amino acid sequences of the variable regions from the mouse monoclonal antibodies to human IAP were applied to the database of amino acid sequences of the antibodies made by Kabat et al. to investigate the homology. The CDR regions were determined 15 based on the homology as shown in Table 1.

Table 1

	<u>Plasmid</u>	<u>SEQ ID NO.</u>	<u>CDR(1)</u>	<u>CDR(2)</u>	<u>CDR(3)</u>
	pGEM-M1L	5	43-58	74-80	113-121
20	pGEM-M1H	6	50-54	69-85	118-125
	pGEM-M2L	7	43-58	74-80	113-121
	pGEM-M2H	8	50-54	69-85	118-125

Example 4 (Identification of Cloned cDNA Expression  
25 Preparation of Chimera MABL-1 antibody and Chimera MABL-2 antibody.)

4.1 Preparation of vectors expressing chimera MABL-1 antibody

5      cDNA clones, pGEM-M1L and pGEM-M1H, encoding the V regions of the L chain and the H chain of the mouse antibody MABL-1, respectively, were modified by the PCR method and introduced into the HEF expression vector (WO92/19759) to prepare vectors expressing chimera MABL-1 antibody.

10     A forward primer MLS (SEQ ID No. 9) for the L chain V region and a forward primer MHS (SEQ ID No. 10) for the H chain V region were designed to hybridize to a DNA encoding the beginning of the leader sequence of each V region and to contain the Kozak consensus sequence (J. Mol. Biol., 196, 947-950, 1987) and HindIII restriction enzyme site. A reverse primer MLAS (SEQ ID No. 11) for the L chain 15 V region and a reverse primer MHAS (SEQ ID No. 12) for the H chain V region were designed to hybridize to a DNA encoding the end of the J region and to contain the splice donor sequence and BamHI restriction enzyme site.

20     100 µl of a PCR solution comprising 10 µl of 10 × PCR Buffer II, 2 mM MgCl<sub>2</sub>, 0.16 mM dNTPs (dATP, dGTP, dCTP and dTTP), 5 units of DNA polymerase AmpliTaq Gold, 0.4 µM each of primers and 8 ng of the template DNA (pGEM-M1L or pGEM-M1H) was preheated at 94°C of the initial temperature for 9 minutes and then heated at 94°C for 1 minute, at 60°C 25 for 1 minute and at 72°C for 1 minute 20 seconds in order. This temperature cycle was repeated 35 times and then the reaction mixture was further heated at 72°C for 10 minutes.

The PCR product was purified using the QIAquick PCR Purification Kit (QIAGEN) and then digested with HindIII and BamHI. The product from the L chain V region was cloned into the HEF expression vector, HEF- $\kappa$  and the product from the H chain V region was cloned into the HEF expression vector, HEF- $\gamma$ . After DNA sequencing, plasmids containing a DNA fragment with a correct DNA sequence are designated as HEF-M1L and HEF-M1H, respectively.

4.2 Preparation of vectors expressing chimera MABL-2

antibodies

Modification and cloning of cDNA were performed in the same manner described in Example 4.1 except for using pGEM-M2L and pGEM-M2H as template DNA instead of pGEM-M1L and pGEM-M1H. After DNA sequencing, plasmids containing DNA fragments with correct DNA sequences are designated as HEF-M2L and HEF-M2H, respectively.

4.3 Transfection to COS7 cells

The aforementioned expression vectors were tested in COS7 cells to observe the transient expression of the chimera MABL-1 and MABL-2 antibodies.

(1) Transfection with genes for the chimera MABL-1 antibody

COS7 cells were co-transformed with the HEF-M1L and HEF-M1H vectors by electroporation using the Gene Pulser apparatus (BioRad). Each DNA (10  $\mu$ g) and 0.8 ml of PBS with  $1 \times 10^7$  cells/ml were added to a cuvette. The mixture was treated with pulse at 1.5 kV, 25  $\mu$ F of electric capacity.

After the restoration for 10 minutes at a room temperature, the electroporated cells were transferred into DMEM culture medium (GIBCO BRL) containing 10%  $\gamma$ -globulin-free fetal bovine serum. After culturing for 72 hours, the supernatant was collected, centrifuged to remove cell fragments and recovered.

5 (2) Transfection with genes coding for the chimera MABL-2 antibody

The co-transfection to COS7 cells with the genes coding for the chimera MABL-2 antibody was carried out in the same manner as described in Example 4.3-(1) except for using the HEF-M2L and HEF-M2H vectors instead of the HEF-M1L and HEF-M1H vectors. The supernatant was recovered in the same manner.

15 4.4 Flow cytometry

Flow cytometry was performed using the aforementioned culture supernatant of COS7 cells to measure binding to the antigen. The culture supernatant of the COS7 cells expressing the chimera MABL-1 antibody or the COS7 cells expressing the chimera MABL-2 antibody, or human IgG antibody (SIGMA) as a control was added to  $4 \times 10^5$  cells of mouse leukemia cell line L1210 expressing human IAP and incubated on ice. After washing, the FITC-labeled anti-human IgG antibody (Cappel) was added thereto. After incubating and washing, the fluorescence intensity thereof was measured using the FACScan apparatus (BECTON DICKINSON).

Since the chimera MABL-1 and MABL-2 antibodies were specifically bound to L1210 cells expressing human IAP, it is confirmed that these chimera antibodies have proper structures of the V regions of the mouse monoclonal 5 antibodies MABL-1 and MABL-2, respectively (Figures 1-3).

Example 5 (Preparation of reconstructed Single chain Fv (scFv) of the antibody MABL-1 and antibody MABL-2)

5.1 Preparation of reconstructed single chain Fv of antibody

10 MABL-1

The reconstructed single chain Fv of antibody MABL-1 was prepared as follows. The H chain V region and the L chain V of antibody MABL-1, and a linker were respectively amplified by the PCR method and were connected to produce 15 the reconstructed single chain Fv of antibody MABL-1. The production method is illustrated in Figure 4. Six primers (A-F) were employed for the production of the single chain Fv of antibody MABL-1. Primers A, C and E have a sense sequence and primers B, D and F have an antisense sequence.

20 The forward primer VHS for the H chain V region (Primer A, SEQ ID No. 13) was designed to hybridize to a DNA encoding the N-terminal of the H chain V region and to contain NcoI restriction enzyme recognition site. The reverse primer VHAS for H chain V region (Primer B, SEQ ID 25 No. 14) was designed to hybridize to a DNA coding the C-terminal of the H chain V region and to overlap with the linker.

The forward primer LS for the linker (Primer C, SEQ ID No. 15) was designed to hybridize to a DNA encoding the N-terminal of the linker and to overlap with a DNA encoding the C-terminal of the H chain V region. The reverse 5 primer LAS for the linker (Primer D, SEQ ID No. 16) was designed to hybridize to a DNA encoding the C-terminal of the linker and to overlap with a DNA encoding the N-terminal of the L chain V region.

The forward primer VLS for the L chain V region (Primer E, SEQ ID No. 17) was designed to hybridize to a DNA encoding the C-terminal of the linker and to overlap with a DNA encoding the N-terminal of the L chain V region. The reverse primer VLAS-FLAG for L chain V region (Primer F, SEQ 10 ID No. 18) was designed to hybridize to a DNA encoding the C-terminal of the L chain V region and to have a sequence encoding the FLAG peptide (Hopp. T. P. et al., Bio/Technology, 6, 1204-1210, 1988), two stop codons and 15 EcoRI restriction enzyme recognition site.

In the first PCR step, three reactions, A-B, C-D and E-F, were carried out and PCR products thereof were purified. Three PCR products obtained from the first PCR step were assembled by their complementarity. Then, the primers A and F were added and the full length DNA encoding the reconstructed single chain Fv of antibody MABL-1 was 20 amplified (Second PCR). In the first PCR, the plasmid pGEM- M1H encoding the H chain V region of antibody MABL-1 (see 25 Example 2), a plasmid pSC-DP1 which comprises a DNA sequence

encoding a linker region comprising: Gly Gly Gly Gly Ser Gly  
Gly Gly Gly Ser Gly Gly Gly Ser (SEQ ID No. 19) (Huston,  
J.S., et al., Proc. Natl. Acad. Sci. USA, 85, 5879-5883,  
1988) and the plasmid pGEM-M1L encoding the L chain V region  
5 of antibody MABL-1 (see Example 2) were employed as  
template, respectively.

50  $\mu$ l of the solution for the first PCR step  
comprises 5  $\mu$ l of 10  $\times$  PCR Buffer II, 2 mM MgCl<sub>2</sub>, 0.16 mM  
dNTPs, 2.5 units of DNA polymerase, AmpliTaq Gold (PERKIN  
10 ELMER), 0.4  $\mu$ M each of primers and 5 ng each of template  
DNA. The PCR solution was preheated at 94°C of the initial  
temperature for 9 minutes and then heated at 94°C for 1  
minute, at 65°C for 1 minute and at 72°C for 1 minute and 20  
seconds in order. This temperature cycle was repeated 35  
15 times and then the reaction mixture was further heated at  
72°C for 7 minutes.

The PCR products A-B (371bp), C-D (63bp) and E-F  
(384bp) were purified using the QIAquick PCR Purification  
Kit (QIAGEN) and were assembled in the second PCR. In the  
20 second PCR, 98  $\mu$ l of a PCR solution comprising 120 ng of the  
first PCR product A-B, 20 ng of the PCR product C-D and 120  
ng of the PCR product E-F, 10  $\mu$ l of 10  $\times$  PCR Buffer II, 2 mM  
MgCl<sub>2</sub>, 0.16 mM dNTPs, 5 units of DNA polymerase AmpliTaq  
Gold (PERKIN ELMER) was preheated at 94°C of the initial  
25 temperature for 8 minutes and then heated at 94°C for 2  
minutes, at 65°C for 2 minutes and at 72°C for 2 minutes in  
order. This temperature cycle was repeated twice and then

0.4  $\mu$ M each of primers A and F were added into the reaction, respectively. The mixture was preheated at 94°C of the initial temperature for 1 minutes and then heated at 94°C for 1 minute, at 65°C for 1 minute and at 72°C for 1 minute and 20 seconds in order. This temperature cycle was repeated 5 35 times and then the reaction mixture was further heated at 72°C for 7 minutes.

A DNA fragment of 843 bp produced by the second PCR was purified and digested by NcoI and EcoRI. The 10 resultant DNA fragment was cloned into pSCFVT7 vector. The expression vector pSCFVT7 contains a pelB signal sequence suitable for E. coli periplasmic expression system (Lei, S.P., et al., J. Bacteriology, 169, 4379-4383, 1987). After the DNA sequencing, the plasmid containing the DNA fragment 15 encoding correct amino acid sequence of the reconstructed single chain Fv of antibody MABL-1 is designated as "pscM1" (see Figure 5). The nucleotide sequence and the amino acid sequence of the reconstructed single chain Fv of antibody MABL-1 contained in the plasmid pscM1 are shown in SEQ ID 20 No. 20.

The pscM1 vector was modified by the PCR method to prepare a vector expressing the reconstructed single chain Fv of antibody MABL-1 in mammalian cells. The resultant DNA fragment was introduced into pCHO1 expression vector. This 25 expression vector, pCHO1, was constructed by digesting DHFR- $\Delta$ E-rvH-PM1-f (WO92/19759) with EcoRI and SmaI to eliminate

the antibody gene and connecting the EcoRI-NotI-BamHI Adapter (Takara Shuzo) thereto.

As a forward primer for PCR, Sal-VHS primer shown in SEQ ID No. 21 was designed to hybridize to a DNA encoding the N-terminal of the H chain V region and to contain SalI restriction enzyme recognition site. As a reverse primer for PCR, FRHlanti primer shown in SEQ ID No. 22 was designed to hybridize to a DNA encoding the end of the first framework sequence.

100  $\mu$ l of PCR solution comprising 10  $\mu$ l of 10  $\times$  PCR Buffer II, 2 mM MgCl<sub>2</sub>, 0.16 mM dNTPs, 5 units of the DNA polymerase, AmpliTaq Gold, 0.4  $\mu$ l M each of primer and 8 ng of the template DNA (pscM1) was preheated at 95°C of the initial temperature for 9 minutes and then heated at 95°C for 1 minute, at 60°C for 1 minute and at 72°C for 1 minute and 20 seconds in order. This temperature cycle was repeated 35 times and then the reaction mixture was further heated at 72°C for 7 minutes.

The PCR product was purified using the QIAquick PCR Purification Kit (QIAGEN) and digested by SalI and MboII to obtain a DNA fragment encoding the N-terminal of the reconstructed single chain Fv of antibody MABL-1. The pscM1 vector was digested by MboII and EcoRI to obtain a DNA fragment encoding the C-terminal of the reconstructed single chain Fv of antibody MABL-1. The SalI-MboII DNA fragment and the MboII-EcoRI DNA fragment were cloned into pCHO1-Igs vector. After DNA sequencing, the plasmid comprising the

desired DNA sequence was designated as "pCHOM1" (see Figure 6). The expression vector, pCH01-Igs, contains a mouse IgG1 signal sequence suitable for the secretion-expression system in mammalian cells (Nature, 322, 323-327, 1988). The 5 nucleotide sequence and the amino acid sequence of the reconstructed single chain Fv of antibody MABL-1 contained in the plasmid pCHOM1 are shown in SEQ ID No. 23.

5.2 Preparation of reconstructed single chain Fv of antibody MABL-2

10 The reconstructed single chain Fv of antibody MABL-2 was prepared in accordance with the aforementioned Example 5.1. Employed in the first PCR step were plasmid pGEM-M2H encoding the H chain V region of MABL-2 (see Example 2) instead of pGEM-M1H and plasmid pGEM-M2L encoding 15 the L chain V region of MABL-2 (see Example 2) instead of pGEM-M1L, to obtain a plasmid pscM2 which comprises a DNA fragment encoding the desired amino acid sequence of the single chain Fv of antibody MABL-2. The nucleotide sequence and the amino acid sequence of the reconstructed single 20 chain Fv of antibody MABL-2 contained in the plasmid pscM2 are shown in SEQ ID No. 24.

The pscM2 vector was modified by the PCR method to 25 prepare a vector, pCHOM2, for the expression in mammalian cells which contains the DNA fragment encoding the correct amino acid sequence of reconstructed the single chain Fv of antibody MABL-2. The nucleotide sequence and the amino acid sequence of the reconstructed single chain Fv of antibody

MABL-2 contained in the plasmid pCHOM2 are shown in SEQ ID No. 25.

### 5.3 Transfection to COS7 cells

5 The pCHOM2 vector was tested in COS7 cells to observe the transient expression of the reconstructed single chain Fv of antibody MABL-2.

10 The COS7 cells were transformed with the pCHOM2 vector by electroporation using the Gene Pulser apparatus (BioRad). The DNA (10  $\mu$ g) and 0.8 ml of PBS with  $1 \times 10^7$  cells/ml were added to a cuvette. The mixture was treated with pulse at 1.5 kV, 25  $\mu$ F of electric capacity.

15 After the restoration for 10 minutes at a room temperature, the electroporated cells were transferred into IMDM culture medium (GIBCO BRL) containing 10% fetal bovine serum. After culturing for 72 hours, the supernatant was collected, centrifuged to remove cell fragments and recovered.

### 5.4 Detection of the reconstructed single chain Fv of antibody MABL-2 in culture supernatant of COS7 cells

20 The existence of the single chain Fv of antibody MABL-2 in the culture supernatant of COS7 cells which had been transfected with the pCHOM2 vector was confirmed by the Western Blotting method.

25 The culture supernatant of COS7 cells transfected with the pCHOM2 vector and the culture supernatant of COS7 cells transfected with the pCHO1 as a control were subjected to SDS electrophoresis and transferred to REINFORCED NC

membrane (Schleicher & Schuell). The membrane was blocked with 5% skim milk (Morinaga Nyu-gyo), washed with 0.05% Tween 20-PBS and mixed with an anti-FLAG antibody (SIGMA). The membrane was incubated at room temperature, washed and mixed with alkaline phosphatase-conjugated mouse IgG antibody (Zymed). After incubating and washing at room temperature, the substrate solution (Kirkegaard Perry Laboratories) was added to develop color (Figure 7).

A FLAG-peptide-specific protein was detected only in the culture supernatant of the pCHOM2 vector-introduced COS7 cells and thus it is confirmed that the reconstructed single chain Fv of antibody MABL-2 was secreted in this culture supernatant.

#### 5.5 Flow cytometry

Flow cytometry was performed using the aforementioned COS7 cells culture supernatant to measure the binding to the antigen. The culture supernatant of the COS7 cells expressing the reconstructed single chain Fv of antibody MABL-2 or the culture supernatant of COS7 cells transformed with pCHO1 vector as a control was added to 2 × 10<sup>5</sup> cells of the mouse leukemia cell line L1210 expressing human Integrin Associated Protein (IAP) or the cell line L1210 transformed with pCOS1 as a control. After incubating on ice and washing, the mouse anti-FLAG antibody (SIGMA) was added. Then the cells were incubated and washed. Then, the FITC labeled anti-mouse IgG antibody (BECTON DICKINSON) was added thereto and the cells were incubated and washed again.

Subsequently, the fluorescence intensity was measured using the FACScan apparatus (BECTON DICKINSON).

Since the single chain Fv of antibody MABL-2 was specifically bound to L1210 cells expressing human IAP, it is confirmed that the reconstructed single chain Fv of antibody MABL-2 has an affinity to human Integrin Associated Protein (IAP) (see Figures 8-11).

#### 5.6 Competitive ELISA

The binding activity of the reconstructed single chain Fv of antibody MABL-2 was measured based on the inhibiting activity against the binding of mouse monoclonal antibodies to the antigen.

The anti-FLAG antibody adjusted to 1  $\mu$ g/ml was added to each well on 96-well plate and incubated at 37°C for 2 hours. After washing, blocking was performed with 1% BSA-PBS. After incubating and washing at a room temperature, the culture supernatant of COS7 cells into which the secretion-type human IAP antigen gene (SEQ ID No. 26) had been introduced was diluted with PBS into twofold volume and added to each well. After incubating and washing at a room temperature, a mixture of 50  $\mu$ l of the biotinized MABL-2 antibody adjusted to 100 ng/ml and 50  $\mu$ l of sequentially diluted supernatant of the COS7 cells expressing the reconstructed single chain Fv of antibody MABL-2 were added into each well. After incubating and washing at a room temperature, the alkaline phosphatase-conjugated streptoavidin (Zymed) was added into each well. After

incubating and washing at a room temperature, the substrate solution (SIGMA) was added and absorbance of the reaction mixture in each well was measured at 405 nm.

5 The results revealed that the reconstructed single chain Fv of antibody MABL-2 (MABL2-scFv) evidently inhibited concentration-dependently the binding of the mouse antibody MABL-2 to human IAP antigen in comparison with the culture supernatant of the PCHO1-introduced COS7 cells as a control (Figure 12). Accordingly, it is suggested that the 10 reconstructed single chain Fv of antibody MABL-2 has the correct structure of each of the V regions from the mouse monoclonal antibody MABL-2.

#### 5.7 Apoptosis-inducing Effect in vitro

15 An apoptosis-inducing action of the reconstructed single chain Fv of antibody MABL-2 was examined by Annexin-V staining (Boehringer Mannheim) using the L1210 cells transfected with human IAP gene, the L1210 cells transfected with the pCOS1 vector as a control and CCRF-CEM cells.

20 To each  $1 \times 10^5$  cells of the above cells was added the culture supernatant of the COS7 cells expressing the reconstructed single chain Fv of antibody MABL-2 or the culture supernatant of COS7 cells transfected with the pCHO1 vector as a control at 50% final concentration and the mixtures were cultured for 24 hours. Then, the Annexin-V 25 staining was performed and the fluorescence intensity was measured using the FACScan apparatus (BECTON DICKINSON).

Results of the Annexin-V staining are shown in Figures 13-18, respectively. Dots in the left-lower region represent living cells and dots in the right-lower region represent cells at the early stage of apoptosis and dots in the right-upper region represent cells at the late stage of apoptosis. The results show that the reconstructed single chain Fv of antibody MABL-2 (MABL2-scFv) remarkably induced cell death of L1210 cells specific to human IAP antigen (Figures 13-16) and that the reconstructed single chain Fv also induced remarkable cell death of CCRF-CEM cells in comparison with the control (Figures 17-18).

#### 5.8 Expression of MABL-2 derived single chain Fv in CHO cells

CHO cells were transfected with the pCHOM2 vector to establish a CHO cell line which constantly expresses the single chain Fv (polypeptide) derived from the antibody MABL-2.

CHO cells were transformed with the pCHOM2 vector by the electroporation using the Gene Pulser apparatus (BioRad). A mixture of DNA (10  $\mu$ g) and 0.7 ml of PBS with CHO cells ( $1 \times 10^7$  cells/ml) was added to a cuvette. The mixture was treated with pulse at 1.5 kV, 25  $\mu$ F of electric capacity. After the restoration for 10 minutes at a room temperature, the electroporated cells were transferred into nucleic acid free  $\alpha$ -MEM medium (GIBCO BRL) containing 10% fetal bovine serum and cultured. The expression of desired protein in the resultant clones was confirmed by SDS-PAGE

and a clone with a high expression level was selected as a cell line producing the single chain Fv derived from the antibody MABL-2. The cell line was cultured in serum-free medium CHO-S-SFM II (GIBCO BRL) containing 10 nM methotrexate (SIGMA). Then, the culture supernatant was collected, centrifuged to remove cell fragments and recovered.

5.9 Purification of MABL-2 derived single chain Fv produced in CHO cells

The culture supernatant of the CHO cell line expressing the single chain Fv obtained in Example 5.8 was concentrated up to twenty times using a cartridge for the artificial dialysis (PAN130SF, ASAHI MEDICALS). The concentrated solution was stored at -20°C and thawed on purification.

Purification of the single chain Fv from the culture supernatant of the CHO cells was performed using three kinds of chromatography, i.e., Blue-sepharose, a hydroxyapatite and a gel filtration.

20 (1) Blue-sepharose column chromatography

The concentrated supernatant was diluted to ten times with 20 mM acetate buffer (pH 6.0) and centrifuged to remove insoluble materials (10000 × rpm, 30 minutes). The supernatant was applied onto a Blue-sepharose column (20 ml) equilibrated with the same buffer. After washing the column with the same buffer, proteins adsorbed in the column were eluted by a stepwise gradient of NaCl in the same buffer,

0.1, 0.2, 0.3, 0.5 and up to 1.0 M. The pass-through fraction and each eluted fraction were analyzed by SDS-PAGE. The fractions in which the single chain Fv were confirmed (the fractions eluted at 0.1 to 0.3M NaCl) were pooled and 5 concentrated up to approximately 20 times using CentriPrep-10 (AMICON).

(2) Hydroxyapatite

The concentrated solution obtained in (1) was diluted to 10 times with 10 mM phosphate buffer (pH 7.0) and 10 applied onto the hydroxyapatite column (20 ml, BIORAD). The column was washed with 60 ml of 10 mM phosphate buffer (pH 7.0). Then, proteins adsorbed in the column were eluted by a linear gradient of sodium phosphate buffer up to 200 mM (see Figure 19). The analysis of each fraction by SDS-PAGE 15 confirmed the single chain Fv in fraction A and fraction B.

(3) Gel filtration

Each of fractions A and B in (2) was separately concentrated with CentriPrep-10 and applied onto TSKgel G3000SWG column (21.5 x 600 mm) equilibrated with 20 mM acetate buffer (pH 6.0) containing 0.15 M NaCl. 20 Chromatograms are shown in Figure 20. The analysis of the fractions by SDS-PAGE confirmed that both major peaks (AI and BI) are of desired single chain Fv. In the gel filtration analysis, the fraction A was eluted at 36 kDa of 25 apparent molecular weight and the fraction B was eluted at 76 kDa. The purified single chain Fvs (AI, BI) were analyzed with 15% SDS polyacrylamide gel. Samples were treated in the

absence or presence of a reductant and the electrophoresis was carried out in accordance with the Laemmli's method. Then the protein was stained with Coomassie Brilliant Blue. As shown in Figure 21, both AI and BI gave a single band at 5 35 kDa of apparent molecular weight, regardless of the absence or presence of the reductant. From the above, it is concluded that AI is a monomer of the single chain Fv and BI is a non-covalently bound dimer of the single chain Fv. The gel filtration analysis of the fractions AI and BI with 10 TSKgel G3000SW column (7.5 × 60 mm) revealed that a peak of the monomer is detected only in the fraction AI and a peak of the dimer is detected only in the fraction BI (Figure 22). The dimer fraction (fraction BI) accounted for 4 period of total single chain Fvs. More than 90% of the dimer in the 15 dimer fraction was stably preserved for more than a month at 4°C .

5.10 Construction of vector expressing single chain Fv derived from antibody MABL-2 in E. coli cell

20 The pscM2 vector was modified by the PCR method to prepare a vector effectively expressing the single chain Fv from the antibody MABL-2 in E. coli cells. The resultant DNA fragment was introduced into pSCFVT7 expression vector.

25 As a forward primer for PCR, Nde-VHSm02 primer shown in SEQ ID No. 27 was designed to hybridize to a DNA encoding the N-terminal of the H chain V region and to contain a start codon and NdeI restriction enzyme recognition site. As a reverse primer for PCR, VLAS primer

shown in SEQ ID No. 28 was designed to hybridize to a DNA encoding the C-terminal of the L chain V region and to contain two stop codons and EcoRI restriction enzyme 5 recognition site. The forward primer, Nde-VHSm02, comprises five point mutations in the part hybridizing to the DNA encoding the N-terminal of the H chain V region for the effective expression in E. coli.

100  $\mu$ l of a PCR solution comprising 10  $\mu$ l of 10 x PCR Buffer #1, 1 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 5 units of KOD DNA polymerase (all from TOYOBO), 1  $\mu$ M of each primer and 100 ng 10 of a template DNA (pscM2) was heated at 98°C for 15 seconds, at 65°C for 2 seconds and at 74°C for 30 seconds in order. This temperature cycle was repeated 25 times.

15 The PCR product was purified using the QIAquick PCR Purification Kit (QIAGEN) and digested by NdeI and EcoRI, and then the resulting DNA fragment was cloned into pSCFVT7 vector, from which pelB signal sequence had been eliminated by the digestion with NdeI and EcoRI. After DNA sequencing, the resulting plasmid comprising a DNA fragment 20 with the desired DNA sequence is designated as "pscM2DEM02" (see Figure 23). The nucleotide sequence and the amino acid sequence of the single chain Fv derived from the antibody MABL-2 contained in the plasmid pscM2DEM02 are shown in SEQ ID No. 29.

25 5.11 Expression of single chain Fv derived from antibody MABL-2 in E. coli cells

E. coli BL21(DE3)pLySS (STRATAGENE) was transformed with pscM2DEM02 vector to obtain a strain of E. coli expressing the single chain Fv derived from antibody MABL-2. The resulting clones were examined for the expression of the desired protein using SDS-PAGE, and a clone with a high expression level was selected as a strain producing the single chain Fv derived from antibody MABL-2.

5 5.12 Purification of single chain Fv derived from antibody MABL-2 produced in E.coli

10 A single colony of E. coli obtained by the transformation was cultured in 3 ml of LB medium at 28°C for 7 hours and then in 70 ml of LB medium at 28°C overnight. This pre-culture was transplanted to 7 L of LB medium and cultured at 28°C with stirring at 300 rpm using the Jar-15 fermenter. When an absorbance of the medium reached O.D.=1.5, the bacteria were induced with 1 mM IPTG and then cultured for 3 hours.

20 The culture medium was centrifuged (10000 × g, 10 minutes) and the precipitated bacteria were recovered. To the bacteria was added 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA, 0.1 M NaCl and 1% Triton X-100 and the bacteria were disrupted by ultrasonication (out put: 4, duty cycle: 70%, 1 minute × 10 times). The suspension of disrupted bacteria was centrifuged (12000 × g, 10 minutes) 25 to precipitate inclusion body. Isolated inclusion body was mixed with 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA, 0.1 M NaCl and 4% Triton X-100, treated by

ultrasonication (out put: 4, duty cycle: 50%, 30 seconds  $\times$  2 times) again and centrifuged (12000  $\times$  g, 10 minutes) to isolate the desired protein as precipitate and to remove containment proteins included in the supernatant.

5                   The inclusion body comprising the desired protein was lysed in 50 mM Tris-HCl buffer (pH 8.0) containing 6 M Urea, 5 mM EDTA and 0.1 M NaCl and applied onto Sephacryl S-300 gel filtration column (5  $\times$  90 cm, Amersham Pharmacia) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM Urea, 5 mM EDTA, 0.1 M NaCl and 10 mM mercaptoethanol at a flow rate of 5 ml/minutes to remove associated single chain Fvs with high-molecular weight. The obtained fractions were analyzed with SDS-PAGE and the fractions with high purity of the protein were diluted with the buffer used in the gel filtration up to  $O.D_{280}=0.25$ . Then, the fractions were dialyzed three times against 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA, 0.1 M NaCl, 0.5 M Arg, 2 mM glutathione in the reduced form and 0.2 mM glutathione in the oxidized form in order for the protein to be refolded. 15                   Further, the fraction was dialyzed three times against 20 mM acetate buffer (pH 6.0) containing 0.15 M NaCl to exchange the buffer.

20                   The dialysate product was applied onto Superdex 200 pg gel filtration column (2.6  $\times$  60 cm, Amersham Pharmacia) equilibrated with 20 mM acetate buffer (pH 6.0) containing 0.15 M NaCl to remove a small amount of high molecular weight protein which was intermolecularly

crosslinked by S-S bonds. As shown in Figure 24, two peaks, major and sub peaks, were eluted after broad peaks which are expectedly attributed to an aggregate with a high molecular weight. The analysis by SDS-PAGE (see Figure 21) and the elution positions of the two peaks in the gel filtration analysis suggest that the major peak is of the monomer of the single chain Fv and the sub peak is of the non-covalently bound dimer of the single chain Fv. The non-covalently bound dimer accounted for 4 percent of total single chain Fvs.

5.13 Apoptosis-inducing activity in vitro of single chain Fv derived from antibody MABL-2

An apoptosis-inducing action of the single chain Fv from antibody MABL-2 (MABL2-scFv) produced by the CHO cells and E. coli was examined according to two protocols by Annexin-V staining (Boehringer Mannheim) using the L1210 cells (hIAP/L1210) into which human IAP gene had been introduced.

In the first protocol sample antibodies at the final concentration of 3  $\mu$ g/ml were added to  $5 \times 10^4$  cells of hIAP/L1210 cell line and cultured for 24 hours. Sample antibodies, i.e., the monomer and the dimer of the single chain Fv of MABL-2 from the CHO cells obtained in Example 5.9, the monomer and the dimer of the single chain Fv of MABL-2 from E. coli obtained in Example 5.12, and the mouse IgG antibody as a control were analyzed. After culturing, the Annexin-V staining was carried out and the fluorescence

intensity thereof was measured using the FACScan apparatus (BECTON DICKINSON).

5 In the second protocol sample antibodies at the final concentration of 3  $\mu$ g/ml were added to  $5 \times 10^4$  cells of hIAP/L1210 cell line, cultured for 2 hours and mixed with anti-FLAG antibody (SIGMA) at the final concentration of 15  $\mu$ g/ml and further cultured for 22 hours. Sample antibodies of the monomer of the single chain Fv of MABL-2 from the CHO cells obtained in Example 5.9 and the mouse IgG antibody as a control were analyzed. After culturing, the Annexin-V staining was carried out and the fluorescence intensity thereof was measured using the FACScan apparatus.

10 Results of the analysis by the Annexin-V staining are shown in Figures 25-31. The results show that the dimers of the single chain Fv polypeptide of MABL-2 produced in the CHO cells and E. coli remarkably induced cell death (Figures 15 26, 27) in comparison with the control (Figure 25), while no apoptosis-inducing action was observed in the monomers of the single chain Fv polypeptide of MABL-2 produced in the CHO cells and E. coli (Figures 28, 29). When anti-FLAG 20 antibody was used together, the monomer of the single chain Fv polypeptide derived from antibody MABL-2 produced in the CHO cells induced remarkably cell death (Figure 31) in comparison with the control (Figure 30).

25 5.14 Antitumor effect of the monomer and the dimer of scFv/CHO polypeptide with a model mouse of human myeloma  
(1) Quantitative measurement of human IgG in mouse serum

Measurement of human IgG (M protein) produced by human myeloma cell and contained in mouse serum was carried out by the following ELISA. 100  $\mu$ L of goat anti-human IgG antibody (BIOSOURCE, Lot#7902) diluted to 1  $\mu$ g/mL with 0.1% bicarbonate buffer (pH 9.6) was added to each well on 96 wells plate (Nunc) and incubated at 4°C overnight so that the antibody was immobilized. After blocking, 100  $\mu$ L of the stepwisely diluted mouse serum or human IgG (CAPPYEL, Lot#00915) as a standard was added to each well and incubated for 2 hours at a room temperature. After washing, 100  $\mu$ L of alkaline phosphatase-labeled anti-human IgG antibody (BIOSOURCE, Lot#6202) which had been diluted to 5000 times was added, and incubation was carried out for 1 hour at a room temperature. After washing, a substrate solution was added. After incubation, absorbance at 405 nm was measured using the MICROPLATE READER Model 3550 (BioRad). The concentration of human IgG in the mouse serum was calculated based on the calibration curve obtained from the absorbance values of human IgG as the standard.

(2) Preparation of antibodies for administration

The monomer and the dimer of the scFv/CHO polypeptide were respectively diluted to 0.4 mg/mL or 0.25 mg/mL with sterile filtered PBS(–) on the day of administration to prepare samples for the administration.

(3) Preparation of a mouse model of human myeloma

A mouse model of human myeloma was prepared as follows. KPMM2 cells passaged *in vivo* (JP-Appl. 7-236475) by

SCID mouse (Japan Clare) were suspended in RPMI1640 medium (GIBCO-BRL) containing 10% fetal bovine serum (GIBCO-BRL) and adjusted to  $3 \times 10^7$  cells/mL. 200  $\mu$ L of the KPMM2 cell suspension ( $6 \times 10^6$  cells/mouse) was transplanted to the 5 SCID mouse (male, 6 week-old) via caudal vein thereof, which had been subcutaneously injected with the asialo GM1 antibody (WAKO JUNYAKU, 1 vial dissolved in 5 mL) a day before the transplantation.

(4) Administration of antibodies

10 The samples of the antibodies prepared in (2), the monomer (250  $\mu$ L) and the dimer (400  $\mu$ L), were administered to the model mice of human myeloma prepared in (3) via caudal vein thereof. The administration was started from three days after the transplantation of KPMM2 cells and was 15 carried out twice a day for three days. As a control, 200  $\mu$ L of sterile filtered PBS(-) was likewise administered twice a day for three days via caudal vein. Each group consisted of seven mice.

(5) Evaluation of antitumor effect of the monomer and the dimer of scFv/CHO polypeptide with the model mouse of human myeloma

20 The antitumor effect of the monomer and the dimer of scFv/CHO polypeptide with the model mice of human myeloma was evaluated in terms of the change of human IgG (M protein) concentration in the mouse serum and survival time 25 of the mice. The change of human IgG concentration was determined by measuring it in the mouse serum collected at

24 days after the transplantation of KPMM2 cells by ELISA described in the above (1). The amount of serum human IgG (M protein) in the serum of the PBS(-)-administered group (control) increased to about 8500  $\mu$ g/mL, whereas the amount of human IgG of the scFv/CHO dimer-administered group was remarkably low, that is, as low as one-tenth or less than that of the control group. Thus, the results show that the dimer of scFv/CHO strongly inhibits the growth of the KPMM2 cells (Figure 32). As shown in Figure 33, a remarkable 5 elongation of the survival time was observed in the scFv/CHO dimer-administered group in comparison with the PBS(-)-administered group.

10 From the above, it is confirmed that the dimer of scFv/CHO has an antitumor effect for the human myeloma model mice. It is considered that the antitumor effect of the 15 dimer of scFv/CHO, the modified antibody of the invention, results from the apoptosis-inducing action of the modified antibody.

#### 5.15 Hemagglutination Test

20 Hemagglutination test and determination of hemagglutination were carried out in accordance with "Immuno-Biochemical Investigation", Zoku-Seikagaku Jikken Koza, edited by the Biochemical Society of Japan, published by Tokyo Kagaku Dojin.

25 Blood was taken from a healthy donor using heparin-treated syringes and washed with PBS(-) three times, and then erythrocyte suspension with a final concentration

of 2% in PBS(-) was prepared. Test samples were the antibody MABL-2, the monomer and the dimer of the single chain Fv polypeptide produced by the CHO cells, and the monomer and the dimer of the single chain Fv polypeptide produced by E. coli, and the control was mouse IgG (ZYMED). For the investigation of the hemagglutination effect, round bottom 96-well plates available from Falcon were used. 50  $\mu$ L per well of the aforementioned antibody samples and 50  $\mu$ L of the 2% erythrocyte suspension were added and mixed in the well.

5 After incubation for 2 hours at 37°C, the reaction mixtures were stored at 4°C overnight and the hemagglutination thereof was determined. As a control, 50  $\mu$ L per well of PBS(-) was used and the hemagglutination test was carried out in the same manner. The mouse IgG and antibody MABL-2

10 were employed at 0.01, 0.1, 1.0, 10.0 or 100.0  $\mu$ g/mL of the final concentration of the antibodies. The single chain Fvs were employed at 0.004, 0.04, 0.4, 4.0, 40.0 or 80.0  $\mu$ g/mL of the final concentration and further at 160.0  $\mu$ g/mL only in the case of the dimer of the polypeptide produced by E. coli.

15 Results are shown in the Table 2. In the case of antibody MABL-2, the hemagglutination was observed at a concentration of more than 0.1  $\mu$ g/mL, whereas no hemagglutination was observed for both the monomer and the dimer of the single chain Fv.

20

Table 2 Hemagglutination Test

	Control	0.01	0.1	1	10	100	µg/mL	
1								
mIgG	-	-	-	-	-	-	-	
MABL-2 (intact)	-	-	+	+++	+++	++		
	Control	0.004	0.04	0.4	4	40	µg/mL	
1								
scFv/CHO monomer	-	-	-	-	-	-	-	
scFv/CHO dimer	-	-	-	-	-	-	-	
	Control	0.004	0.04	0.4	4	40	160	µg/mL
1								
scFv/E.coli monomer	-	-	-	-	-	-	-	
scFv/E.coli dimer	-	-	-	-	-	-	-	

Example 6 Modified antibody sc(Fv)<sub>2</sub> comprising two H chain V regions and two L chain V regions and antibody MABL-2 scFvs having linkers with different length

5 6.1 Construction of plasmid expressing antibody MABL-2

sc(Fv)<sub>2</sub>

For the preparation of a plasmid expressing the modified antibody [sc(Fv)<sub>2</sub>] which comprises two H chain V regions and two L chain V regions derived from the antibody MABL-2, the aforementioned pCHOM2, which comprises the DNA encoding scFv derived from the MABL-2 described above, was modified by the PCR method as mentioned below and the resulting DNA fragment was introduced into pCHOM2.

15 Primers employed for the PCR are EF1 primer (SEQ ID NO: 30) as a sense primer, which is designed to hybridize

to a DNA encoding EF1 $\alpha$ , and an antisense primer (SEQ ID NO: 19), which is designed to hybridize to the DNA encoding C-terminal of the L chain V region and to contain a DNA sequence coding for a linker region, and VLLAS primer 5 containing SalI restriction enzyme recognition site (SEQ ID NO 31).

100  $\mu$ l of the PCR solution comprises 10  $\mu$ l of 10  $\times$  PCR Buffer #1, 1 mM MgCl<sub>2</sub>, 0.2 mM dNTPs (dATP, dGTP, dCTP and dTTP), 5 units of KOD DNA polymerase (Toyobo, Inc.), 1  $\mu$ M of each primer and 100 ng of the template DNA (pCHOM2). The PCR solution was heated at 94°C for 30 seconds, at 50°C for 30 seconds and at 74°C for 1 minute in order. This temperature cycle was repeated 30 times.

The PCR product was purified using the QIAquick PCR Purification Kit (QIAGEN) and digested by SalI. The resultant DNA fragment was cloned into pBluescript KS<sup>+</sup> vector (Toyobo, Inc.). After DNA sequencing, a plasmid comprising the desired DNA sequence was digested by SalI and the obtained DNA fragment was connected using Rapid DNA Ligation Kit (BOEHRINGER MANNHEIM) to pCHOM2 digested by SalI. After DNA sequencing, a plasmid comprising the desired DNA sequence is designated as "pCHOM2(Fv)<sub>2</sub>" (see Figure 34). The nucleotide sequence and the amino acid sequence of the antibody MABL-2 sc(Fv)<sub>2</sub> region contained in the plasmid pCHOM2(Fv)<sub>2</sub> are shown in SEQ ID No. 32.

6.2 Preparation of Plasmid expressing antibody MABL-2 scFvs having linkers with various length

5 The scFvs containing linkers with different length and the V regions which are designed in the order of [H chain]-[L chain] (hereinafter "HL") or [L chain]-[H chain] (hereinafter "LH") were prepared using, as a template, cDNAs encoding the H chain and the L chain derived from the MABL-2 as mentioned below.

To construct HL type scFv the PCR procedure was carried out using pCHOM2(Fv)<sub>2</sub> as a template. In the PCR step, a pair of CFHL-F1 primer (SEQ ID NO: 33) and CFHL-R2 primer (SEQ ID NO: 34) or a pair of CFHL-F2 primer (SEQ ID NO: 35) and CFHL-R1 primer (SEQ ID NO: 36) and KOD polymerase were employed. The PCR procedure was carried out by repeating 30 times the temperature cycle consisting of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute in order to produce a cDNA for the H chain containing a leader sequence at 5'-end or a cDNA for the L chain containing FLAG sequence at 3'-end thereof. The resultant cDNAs for the H chain and the L chain were mixed and PCR was carried out by repeating 5 times the temperature cycle consisting of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute in order using the mixture as templates and the KOD polymerase. To the reaction mixture were added CFHL-F1 and CFHL-R1 primers and then the PCR reaction was performed by repeating 30 times of the aforementioned temperature cycle to produce a cDNA for HL-0 type without a linker.

To construct LH type scFv, the PCR reaction was carried out using, as a template, pGEM-M2L and pGEM-M2H which contain cDNAs encoding the L chain V region and the H chain V region from the antibody MABL-2, respectively (see 5 JP- Appl. 11-63557). A pair of T7 primer (SEQ ID NO: 37) and CFLH-R2 primer (SEQ ID NO: 38) or a pair of CFLH-F2 primer (SEQ ID NO: 39) and CFLH-R1 (SEQ ID NO: 40) and the KOD polymerase (Toyobo Inc.) were employed. The PCR reaction was performed by repeating 30 times the temperature cycle 10 consisting of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute in sequential order to produce a cDNA of an L chain containing a leader sequence at 5'-end or a cDNA of an H chain containing FLAG sequence at 3'-end thereof. The resultant cDNAs of the L chain and the H chain were 15 mixed and PCR was carried out using this mixture as templates and the KOD polymerase by repeating 5 times the temperature cycle consisting of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute in order. To the reaction mixture were added T7 and CFLH-R1 primers and the 20 reaction was performed by repeating 30 times of the aforementioned temperature cycle. The reaction product was used as a template and PCR was carried out using a pair of CFLH-F4 primer (SEQ ID NO: 41) and CFLH-R1 primer by repeating 30 times the temperature cycle consisting of 94°C 25 for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute in order to produce a cDNA of LH-0 type without a linker.

The resultant cDNAs of LH-0 and HL-0 types were digested by EcoRI and BamHI restriction enzymes (Takara Shuzo) and the digested cDNAs were introduced into an expression plasmid INPEP4 for mammalian cells using Ligation High (Toyobo Inc.), respectively. Competent E. coli JM109 (Nippon Gene) was transformed with each plasmid and the desired plasmids were isolated from the transformed E. coli using QIAGEN Plasmid Maxi Kit (QIAGEN). Thus plasmids pCF2LH-0 and pCF2HL-0 were prepared.

To construct the expression plasmids of HL type containing linkers with different size, pCF2HL-0, as a template, and CFHL-X3 (SEQ ID NO: 42), CFHL-X4 (SEQ ID NO: 43), CFHL-X5 (SEQ ID NO: 44), CFHL-X6 (SEQ ID NO: 45) or CFHL-X7 (SEQ ID NO: 46), as a sense primer, and BGH-1 (SEQ ID NO: 47) primer, as an antisense primer, which is complementary with the vector sequence were employed. PCR reaction was carried out using the KOD polymerase by repeating 30 times the temperature cycle consisting of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute in order and the reaction products were digested by restriction enzymes XhoI and BamHI (Takara Shuzo). The digested fragments were introduced between XhoI and BamHI sites in the pCF2HL-0 using Ligation High (Toyobo Inc.), respectively. Competent E. coli JM109 was transformed with each plasmid and the desired plasmids were isolated from the transformed E. coli by using Qiagen Plasmid Maxi kit. Thus

expression plasmids pCF2HL-3, pCF2HL-4, pCF2HL-5, pCF2HL-6 and pCF2HL-7 were prepared.

To construct expression plasmid for the transient expression in COS7 cells the plasmids pCF2HL-0, pCF2HL-3, pCF2HL-4, pCF2HL-5, pCF2HL-6 and pCF2HL-7 were digested by restriction enzymes EcoRI and BamHI (Takara Shuzo) and the resultant fragments of approximately 800 bp were purified with agarose gel electrophoresis. The obtained fragments were introduced between EcoRI and BamHI sites in an expression plasmid pCOS1 for the expression in mammalian cells by using Ligation High (Toyobo Inc.), respectively.

Competent E. coli DH5 $\alpha$  (Toyobo Inc.) was transformed with each plasmid and the desired plasmids were isolated from the transformed E. coli using Qiagen Plasmid Maxi kit. Thus the expression plasmids CF2HL-0/pCOS1, CF2HL-3/pCOS1, CF2HL-4/pCOS1, CF2HL-5/pCOS1, CF2HL-6/pCOS1 and CF2HL-7/pCOS1 were prepared.

As a typical example of these plasmids, the construction of the plasmid CF2HL-0/pCOS1 is illustrated in Figure 35 and the nucleotide sequence and the amino acid sequence of MABL2-scFv <HL-0> contained in the plasmid are shown in SEQ ID NO. 48. Nucleotide sequences and amino acid sequences of the linker regions in these plasmids are also shown in Figure 36.

To construct the expression plasmids of LH type containing linkers with different size, pCF2LH-0, as a template, and CFLH-X3 (SEQ ID NO: 49), CFLH-X4 (SEQ ID NO:

50), CFLH-X5 (SEQ ID NO: 51), CFLH-X6 (SEQ ID NO: 52) or  
CFLH-X7 (SEQ ID NO: 53), as a sense primer, and BGH-1  
primer, as an antisense primer, which is complementary with  
the vector sequence were employed. PCR reaction was carried  
5 out using the KOD polymerase by repeating 30 times the  
temperature cycle consisting of 94°C for 30 seconds, 60°C  
for 30 seconds and 72°C for 1 minute in order and the  
reaction products were digested by restriction enzymes XhoI  
and BamHI. The digested fragments were introduced into the  
10 pCF2LH-0 between XhoI and BamHI sites using Ligation High,  
respectively. Competent E. coli DH5 $\alpha$  (Toyobo Inc.) was  
transformed with each plasmid and the desired plasmids were  
isolated from the transformed E. coli using Qiagen Plasmid  
Maxi kit. Thus expression plasmids pCF2LH-3, pCF2LH-4,  
15 pCF2LH-5, pCF2LH-6 and pCF2LH-7 were prepared.

20 To construct expression plasmid for the transient  
expression in COS7 cells the plasmids pCF2LH-0, pCF2LH-3,  
pCF2LH-4, pCF2LH-5, pCF2LH-6 and pCF2LH-7 were digested by  
restriction enzymes EcoRI and BamHI (Takara Shuzo) and the  
resultant fragments of approximately 800 bp were purified  
with agarose gel electrophoresis. The obtained fragments  
25 were introduced between XhoI and BamHI sites in an  
expression plasmid pCOS1 for the expression in mammalian  
cells by using the Ligation High, respectively. Competent E.  
coli DH5 $\alpha$  (Toyobo Inc.) was transformed with each plasmid  
and the desired plasmids were isolated from the transformed  
E. coli using the Qiagen Plasmid Maxi kit. Consequently, the

expression plasmids CF2LH-0/pCOS1, CF2LH-3/pCOS1, CF2LH-4/pCOS1, CF2LH-5/pCOS1, CF2LH-6/pCOS1 and CF2LH-7/pCOS1 were prepared.

As a typical example of these plasmids, the  
5 construction of the plasmid CF2LH-0/pCOS1 is illustrated in  
Figure 37 and the nucleotide sequence and the amino acid  
sequence of MABL2-scFv <LH-0> contained in the plasmid are  
shown in SEQ ID No. 54. Nucleotide sequences and amino acid  
sequences of the linker regions in these plasmids are also  
10 shown in Figure 38.

### 6.3 Expression of scFvs and sc(Fv)<sub>2</sub> in COS7 cells

#### (1) Preparation of culture supernatant using serum-containing culture medium

The HL type and LH type of scFvs and sc(Fv)<sub>2</sub> were  
15 transiently expressed in COS7 cells (JCRB9127, Japan Health  
Sciences Foundation). COS7 cells were subcultured in DMEM  
media (GIBCO BRL) containing 10% fetal bovine serum  
(HyClone) at 37°C in carbon dioxide atmosphere incubator.  
The COS7 cells were transfected with CF2HL-0, 3 ~ 7/pCOS1,  
20 or CF2LH-0, 3 ~ 7/pCOS1 prepared in Example 6.2 or  
pCHOM2(Fv)<sub>2</sub> vectors by electroporation using the Gene Pulser  
apparatus (BioRad). The DNA (10 µg) and 0.25 ml of 2 × 10<sup>7</sup>  
cells/ml in DMEM culture medium containing 10% FBS and 5 mM  
BES (SIGMA) were added to a cuvette. After standing for 10  
25 minutes the mixtures were treated with pulse at 0.17kV,  
950µF of electric capacity. After the restoration for 10  
minutes at room temperature, the electroporated cells were

transferred into the DMEM culture medium (10%FBS) in 75 cm<sup>3</sup> flask. After culturing for 72 hours, the culture supernatant was collected and centrifuged to remove cell fragments. The culture supernatant was subjected to the filtration using 5 0.22 µm bottle top filter (FALCON) to obtain the culture supernatant (hereinafter "CM").

(2) Preparation of culture supernatant using serum-free culture medium

Cells transfected in the same manner as (1) were 10 transferred to the DMEM medium (10% FBS) in 75 cm<sup>3</sup> flask and cultured overnight. After the culture, the supernatant was discarded and the cells were washed with PBS and then added to CHO-S-SFM II medium (GIBCO BRL). After culturing for 72 hours, the culture supernatant was collected, centrifuged to 15 remove cell fragments and filtered using 0.22 µm bottle top filter (FALCON) to obtain CM.

6.4 Detection of scFvs and sc(Fv)<sub>2</sub> in CM of COS7

The various MABL2-scFVs and sc(Fv)<sub>2</sub> in CM of COS7 20 prepared in the aforementioned Example 6.3 (2) were detected by Western Blotting method.

Each CM of COS7 was subjected to SDS-PAGE 25 electrophoresis and transferred to REINFORCED NC membrane (Schleicher & Schuell). The membrane was blocked with 5% skim milk (Morinaga Nyu-gyo) and washed with TBS. Then an anti-FLAG antibody (SIGMA) was added thereto. The membrane was incubated at room temperature and washed. A peroxidase labeled mouse IgG antibody (Jackson Immuno Research) was

added. After incubating and washing at room temperature, the substrate solution (Kirkegaard Perry Laboratories) was added to develop color (Figure 39).

#### 6.5 Flow cytometry

Flow cytometry was performed using the culture supernatants of COS7 cells prepared in Example 6.3 (1) to measure the binding of the MABL2-scFVs and sc(Fv)<sub>2</sub> to human Integrin Associated Protein (IAP) antigen. The culture supernatants to be tested or a culture supernatant of COS7 cells as a control was added to  $2 \times 10^5$  cells of the mouse leukemia cell line L1210 expressing human IAP. After incubating on ice and washing, 10  $\mu$ g/mL of the mouse anti-FLAG antibody (SIGMA) was added and then the cells were incubated and washed. Then, the FITC labeled anti-mouse IgG antibody (BECTON DICKINSON) was added thereto and the cells were incubated and washed again. The fluorescence intensity was measured using the FACScan apparatus (BECTON DICKINSON). The results of the flow cytometry show that the MABL2-scFVs having linkers with different length and the sc(Fv)<sub>2</sub> in the culture supernatants of COS7 have high affinity to human IAP (see Figures 40a and 40b).

#### 6.6 Apoptosis-inducing Effect in vitro

An apoptosis-inducing action of the culture supernatants of COS7 prepared in Example 6.3 (1) was examined by Annexin-V staining (Boehringer Mannheim) using the L1210 cells transfected with human IAP gene (hIAP/L1210).

To  $5 \times 10^4$  cells of the hIAP/L1210 cells were added the culture supernatants of COS7 cells transfected with each vectors or a culture supernatant of COS7 cells as a control at 10% of the final concentration and the mixtures were cultured for 24 hours. Then, the Annexin-V/PI staining was performed and the fluorescence intensity was measured using the FACScan apparatus (BECTON DICKINSON). The results revealed that scFvs <HL3, 4, 6, 7, LH3, 4, 6, 7> and sc(Fv)<sub>2</sub> in CM of COS7 induced remarkable cell death of hIAP/L1210 cells. These results are shown in Figure 41.

10 15 6.7 Construction of vectors for the expression of scFvs and sc(Fv)<sub>2</sub> in CHO cells

To isolate and purify MABL2-scFvs and sc(Fv)<sub>2</sub> from culture supernatant, the expression vectors for expressing in CHO cells were constructed as below.

20 The EcoRI-BamHI fragments of pCF2HL-0, 3 ~ 7, and pCF2LH-0, 3 ~ 7 prepared in Example 6.2 were introduced between EcoRI and BamHI sites in an expression vector pCHO1 for CHO cells using the Ligation High. Competent E. coli DH5 $\alpha$  was transformed with them. The plasmids were isolated from the transformed E. coli using QIAGEN Plasmid Midi kit (QIAGEN) to prepare expression plasmids pCHOM2HL-0, 3 ~ 7, and pCHOM2LH-0, 3 ~ 7.

25 6.8 Production of CHO cells expressing MABL2-scFvs <HL-0, 3 ~ 7>, MABL2-scFvs <LH-0, 3 ~ 7> and sc(Fv)<sub>2</sub> and preparation of the culture supernatants thereof

CHO cells were transformed with each of the expression plasmids pCHOM2HL-0, 3 ~ 7, and pCHOM2LH-0, 3 ~ 7, constructed in Example 6.7 and pCHOM2(Fv)<sub>2</sub> vector to prepare the CHO cells constantly expressing each modified antibody. As a typical example thereof, the production of the CHO cells constantly expressing MABL2-scFv <HL-5> or sc(Fv)<sub>2</sub> is illustrated as follows.

The expression plasmids pCHOM2HL-5 and pCHOM2(Fv)<sub>2</sub> were linearized by digesting with a restriction enzyme PvuI and subjected to transfection to CHO cells by electroporation using Gene Pulser apparatus (BioRad). The DNA (10  $\mu$ g) and 0.75 ml of PBS with  $1 \times 10^7$  cells/ml were added to a cuvette and treated with pulse at 1.5 kV, 25  $\mu$ F of electric capacity. After the restoration for 10 minutes at room temperature, the electroporated cells were transferred into nucleic acid-containing  $\alpha$ -MEM culture medium (GIBCO BRL) containing 10% fetal bovine serum and cultured. After culturing overnight, the supernatant was discarded. The cells were washed with PBS and added to nucleic acid-free  $\alpha$ -MEM culture medium (GIBCO BRL) containing 10% fetal bovine serum. After culturing for two weeks, the cells were cultured in a medium containing 10 nM (final concentration) methotrexate (SIGMA), then 50 nM and 100 nM methotrexate. The resultant cells were cultured in serum-free CHO-S-SFM II medium (GIBCO BRL) in a roller bottle. The culture supernatant was collected, centrifuged

to remove cell fragments and filtered using a filter with 0.22  $\mu$ m of pore size to obtain CM, respectively.

According to the above, CHO cells which constantly express MABL2-scFvs <HL-0, -3, -4, -6, -7> and <LH-0, -3, -4, -5, -6, -7> and CMs thereof were obtained.

#### 6.9 Purification of dimer of MABL2-scFv <HL-5> and sc(Fv)<sub>2</sub>

The MABL2-scFv <HL-5> and the sc(Fv)<sub>2</sub> were purified from CMs prepared in Example 6.8 by two types of purification method as below.

##### 10 <Purification Method 1>

HL-5 and sc(Fv)<sub>2</sub> were purified by the anti-FLAG antibody affinity column chromatography utilizing the FLAG sequence located at C-terminal of the polypeptides and by gel filtration. One liter of CM as obtained in 6.8 was applied onto a column (7.9ml) prepared with anti-FLAG M2 Affinity gel (SIGMA) equilibrated with 50 mM Tris-HCl buffer (TBS, pH 7.5) containing 150 mM NaCl. After washing the column with TBS, the scFv was eluted by 0.1 M glycine-HCl buffer, pH 3.5. The resultant fractions were analyzed by SDS-PAGE and the elution of the scFv was confirmed. The scFv fraction was mixed with Tween 20 up to 0.01% of the final concentration and concentrated using Centricon-10 (MILIPORE). The concentrate was applied onto TSKgel G3000SWG column (7.5 x 600 mm) equilibrated with 20 mM acetate buffer (pH 6.0) containing 150 mM NaCl and 0.01% Tween 20. At 0.4 mL/minute of the flow rate, the scFv was detected by the absorption at 280 nm. The HL-5 was eluted as the major

fraction in the position of the dimer and the sc(Fv)<sub>2</sub> was eluted in the position of the monomer.

**<Purification Method 2>**

5        HL-5 and sc(Fv)<sub>2</sub> were purified using three steps comprising ion exchange chromatography, hydroxyapatite and gel filtration. In the ion exchange chromatography, Q sepharose fast flow column (Pharmacia) was employed for HL-5 and SP-sepharose fast flow column was employed for sc(Fv)<sub>2</sub>. In and after the second step, HL-5 and sc(Fv)<sub>2</sub> were 10        processed by the same procedure.

**First step for HL-5**

15        CM of HL-5 was diluted to two times with 20 mM Tris-HCl buffer (pH 9.0) containing 0.02% Tween 20 and then the pH was adjusted to 9.0 with 1 M Tris. The solution was applied onto Q Sepharose fast flow column equilibrated with 20 mM Tris-HCl buffer (pH 8.5) containing 0.02% Tween 20. A polypeptide adsorbed to the column was eluted by a linear gradient of NaCl in the same buffer, from 0.1 to 0.55 M. Monitoring the eluted fractions by SDS-PAGE, the fractions 20        containing HL-5 were collected and subjected to hydroxyapatite of the second step.

**First step for sc(Fv)<sub>2</sub>**

25        CM of the sc(Fv)<sub>2</sub> was diluted to two times with 20mM acetate buffer (pH 5.5) containing 0.02% Tween 20 and its pH was adjusted to 5.5 with 1 M acetic acid. The solution was applied onto a SP-Sepharose fast flow column equilibrated with 20 mM acetate buffer (pH 5.5) containing

0.02% Tween 20. A polypeptide adsorbed to the column was eluted by a linear gradient of NaCl in the buffer, from 0 to 0.5 M. Monitoring the eluted fractions by SDS-PAGE, the fractions containing the sc(Fv)<sub>2</sub> were collected and 5 subjected to hydroxyapatite of the second step.

Second step: Hydroxyapatite chromatography of HL-5 and sc(Fv)<sub>2</sub>

The fractions of HL-5 and sc(Fv)<sub>2</sub> obtained in the first step were separately applied onto the hydroxyapatite 10 column (Type I, BIORAD) equilibrated with 10 mM phosphate buffer containing 0.02% Tween 20, pH 7.0. After washing the column with the same buffer, polypeptides adsorbed to the column were eluted by a linear gradient of the phosphate buffer up to 0.5 M. Monitoring the eluted fractions by SDS- 15 PAGE, the fractions containing the desired polypeptides were collected.

Third step: Gel filtration of HL-5 and sc(Fv)<sub>2</sub>

Each fraction obtained at the second step was 20 separately concentrated with CentriPrep-10 (MILIPORE) and applied onto a Superdex 200 column (2.6 x 60 cm, Pharmacia) equilibrated with 20 mM acetate buffer (pH 6.0) containing 0.02% Tween 20 and 0.15 M NaCl. HL-5 was eluted in the position of the dimer, and sc(Fv)HL-5 and sc(Fv)<sub>2</sub> were 25 eluted in the position of the monomer as a major peak respectively.

Since the monomer of HL-5 was hardly detected by both purification methods, it is proved that the dimers of

single chain Fvs are formed in high yields when the linker for the single chain Fv contains around 5 amino acids. Furthermore, the dimer of HL-5 and the sc(Fv)<sub>2</sub> were stably preserved for a month at 4°C after the purification.

5 6.10 Evaluation of the binding activity of purified dimer of scFv <HL-5> and sc(Fv)<sub>2</sub> against antigen

Flow cytometry was performed using the purified dimer of MABL2-scFv <HL-5> and the purified sc(Fv)<sub>2</sub> in order to evaluate the binding to human Integrin Associated Protein (IAP) antigen. 10 $\mu$ g/ml of the purified dimer of MABL2-scFv <HL-5>, the purified sc(Fv)<sub>2</sub>, the antibody MABL-2 as a positive control or a mouse IgG (Zymed) as a negative control was added to  $2 \times 10^5$  cells of the mouse leukemia cell line L1210 expressing human IAP (hIAP/L1210) or the cell line L1210 transformed with pCOS1 (pCOS1/L1210) as a control. After incubating on ice and washing, 10 $\mu$ g/mL of the mouse anti-FLAG antibody (SIGMA) was added and then the cells were incubated and washed. FITC labeled anti-mouse IgG antibody (BECTON DICKINSON) was added thereto and the cells were incubated and washed again. Then the fluorescence intensity was measured using the FACScan apparatus (BECTON DICKINSON).

20 Since the purified dimer of MABL2-scFv <HL-5> and the purified sc(Fv)<sub>2</sub> were specifically bound to hIAP/L1210 cells, it is confirmed that the dimer of scFv <HL-5> and the sc(Fv)<sub>2</sub> have high affinity to human IAP (see Figure 42).

6.11 Apoptosis-inducing activity in vitro of purified dimer of scFv <HL-5> and sc(Fv)<sub>2</sub>

An apoptosis-inducing action of the purified dimer of MABL2-scFv <HL-5> and the purified sc(Fv)<sub>2</sub> were examined 5 by Annexin-V staining (Boehringer Mannheim) using the L1210 cells (hIAP/L1210) in which human IAP gene had been introduced and cells of human leukemic cell line CCRF-CEM.

Different concentrations of the purified dimer of MABL2-scFv <HL-5>, the purified MABL2-sc(Fv)<sub>2</sub>, the antibody 10 MABL-2 as a positive control or a mouse IgG as a negative control were added to  $5 \times 10^4$  cells of hIAP/L1210 cell line or  $1 \times 10^5$  cells of CCRF-CEM cell line. After culturing for 24 hours, the Annexin-V staining was carried out and the 15 fluorescence intensity thereof was measured using the FACScan apparatus (BECTON DICKINSON). As a result the dimer of MABL2-scFv <HL-5> and the MABL2-sc(Fv)<sub>2</sub> remarkably induced cell death of hIAP/L1210 and CCRF-CEM in concentration-dependent manner (see Figure 43). As a result it was shown that the dimer of MABL2-scFv <HL-5> and MABL2- 20 sc(Fv)<sub>2</sub>, had improved efficacy of inducing apoptosis compared with original antibody MABL-2.

6.12 Hemagglutination Test of the purified dimer of scFv <HL-5> and the sc(Fv)<sub>2</sub>

Hemagglutination test was carried out using 25 different concentrations of the purified dimer of scFv <HL-5> and the purified sc(Fv)<sub>2</sub> in accordance with Example 5.15.

The hemagglutination was observed with the antibody MABL-2 as a positive control, whereas no hemagglutination was observed with both the single chain antibody MABL2-sc(Fv)<sub>2</sub> and the MABL2-scFv <HL-5>. Further, 5 there was no substantial difference in the hemagglutination between two buffers employed with the antibody MABL-2. These results are shown in Table 3.

## Hemagglutination Test

TABLE 3

Diluent : PBS

( $\mu\text{g/ml}$ )

Diluent : PBS										Diluent : Acetate Buffer							
	cont	28.9	14.45	7.225	3.6125	1.8063	0.9031	0.4516	0.2258	0.1129	0.0564	0.0282	0.0141	0.0071	0.0035	0.0018	
MABL2-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
sc(Fv) <sub>2</sub>																	
	cont	28.0	14.0	7.0	3.5	1.75	0.875	0.4375	0.2188	0.1094	0.0547	0.0273	0.0137	0.0068	0.0034	0.0017	
MABL2-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
sc(Fv)																	
<HL5>																	
	cont	80	40	20	10	5	2.5	1.25	0.625	0.3125	0.1563	0.0781	0.0391	0.0195	0.0098	0.0049	
MABL2	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	
(intact)																	
mlgG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

6.13 Antitumor effect of the purified dimer of scFv <HL-5>  
and the sc(Fv)<sub>2</sub> for a model mouse of human myeloma

The antitumor effects were tested for the dimer of scFv <HL-5> and the sc(Fv)<sub>2</sub> prepared and purified in Examples 6.8 and 6.9. The test was performed by using the mouse model for human myeloma produced in Example 5.1 and determining the amount of M protein produced by human myeloma cells in the mouse serum using ELISA and examining survival time of the mice. Then, the antitumor effects of the dimer of scFv <HL-5> and the sc(Fv)<sub>2</sub> were evaluated in terms of the change of the amount of M protein in the mouse serum and the survival time of the mice.

In the test, the HL-5 and the sc(Fv)<sub>2</sub> were employed as a solution at 0.01, 0.1 or 1 mg/mL in vehicle consisting of 150 mM NaCl, 0.02% Tween and 20 mM acetate buffer, pH 6.0 and administered to the mice at 0.1, 1 or 10 mg/kg of dosage. Control group of mice were administered only with the vehicle.

The mouse serum was gathered 26 days after the transplantation of the human myeloma cells and the amount of M protein in the serum was measured using ELISA according to Example 5.14. As a result, the amount of M protein in the serum of both mice groups administered with HL-5, the dimer and the sc(Fv)<sub>2</sub> decreased in dose-dependent manner (see Figure 44). Furthermore, a significant elongation of the survival time was observed in both groups administered with the HL-5 (Figure 45) and with the sc(Fv)<sub>2</sub> (Figure 46) in

comparison with the control group administered with the vehicle. These results show that the HL-5 and the sc(Fv), of the invention have excellent antitumor effect in vivo.

Example 7

5 Single chain Fv comprising H chain V region and L chain V region of human antibody 12B5 against human MPL

A DNA encoding V regions of human monoclonal antibody 12B5 against human MPL was constructed as follows:

7.1 Construction of a gene encoding H chain V region of 12B5

10 The gene encoding H chain V region of human antibody 12B5 binding to human MPL was designed by connecting the nucleotide sequence of the gene thereof (SEQ ID NO: 55) at the 5'-end to the leader sequence (SEQ ID NO: 56) originated from human antibody gene (Eur. J. Immunol. 1996; 26: 63-69).

15 The designed nucleotide sequence was divided into four oligonucleotides having overlapping sequences of 15 bp each (12B5VH-1, 12B5VH-2, 12B5VH-3, 12B5VH-4). 12B5VH-1 (SEQ ID NO: 57) and 12B5VH-3 (SEQ ID NO: 59) were synthesized in the sense direction, and 12B5VH-2 (SEQ ID NO: 58) and 12B5VH-4 (SEQ ID NO: 60) in the antisense direction, respectively.

20 After assembling each synthesized oligonucleotide by respective complementarity, the outside primers (12B5VH-S and 12B5VH-A) were added to amplify the full length of the gene. 12B5VH-S (SEQ ID NO: 61) was designed to hybridize to 25 5'-end of the leader sequence by the forward primer and to have Hind III restriction enzyme recognition site and Kozak sequence, and 12B5VH-A (SEQ ID NO: 62) was designed to

hybridize to the nucleotide sequence encoding C-terminal of H chain V region by the reverse primer and to have a splice donor sequence and BamHI restriction enzyme recognition site, respectively.

5        100 $\mu$ l of the PCR solution containing 5 $\mu$ l of 10 x PCR Gold Buffer II, 1.5mM MgCl<sub>2</sub>, 0.08mM dNTPs (dATP, dGTP, dCTP, dTTP), 5 units of DNA-polymerase AmpliTaq Gold (all by PERKIN ELMER) and each 2.5 $\mu$ l of each synthesized oligonucleotide (12B5VH-1 to -4) was heated at 94°C of the initial temperature for 9 minutes, at 94°C for 2 minutes, at 10 55°C for 2 minutes and 72°C for 2 minutes. After repeating the cycle two times each 100 pmole of external primer 12B5VH-S and 12B5VH-A was added. The mixture was subjected to the cycle consisting of at 94°C for 30 seconds, at 55°C 15 for 30 seconds and 72°C for 1 minute 35 times and heated at 72°C for further 5 minutes.

20        The PCR product was purified by 1.5% low-melting-temperature agarose gel (Sigma), digested by restriction enzymes BamHI and Hind III, and cloned into expression vector HEF-g $\gamma$ 1 for human H chain. After determining the DNA sequence the plasmid containing the correct DNA sequence was named HEF-12B5H-g $\gamma$ 1.

25        The HEF-12B5H-g $\gamma$ 1 was digested by restriction enzymes EcoRI and BamHI to produce the gene encoding 12B5VH which was then cloned into an expression vector pCOS-Fd for human Fab H chain to produce pFd-12B5H. The expression vector for human Fab H chain was constructed by amplifying the DNA (SEQ

5 ID NO: 63) containing the intron region existing between the genes encoding human antibody H chain V region and the constant region, and the gene encoding a part of the constant region of human H chain by PCR, and inserting the  
10 PCR product into animal cell expression vector pCOS1. The human H chain constant region was amplified for the gene under the same conditions mentioned above using as the template HEF- $\gamma$ 1, as the forward primer G1CH1-S (SEQ ID NO: 64) which was designed to hybridize to 5'-end sequence of intron 1 and to have restriction enzyme recognition sites EcoRI and BamHI and as the reverse primer G1CH1-A (SEQ ID NO: 65) which was designed to hybridize to 3'-end DNA of human H chain constant region CH1 domain and to have a sequence encoding a part of hinge region, two stop codons  
15 and restriction enzyme recognition site Bgl II.

The nucleotide sequence and amino acid sequence of the reconstructed 12B5H chain variable region which were included in plasmids HEF-12B5H- $\gamma$ 1 and pFd-12B5H are shown in SEQ ID NO: 66.

20 7.2 Construction of the gene encoding 12B5 L chain V region

The gene encoding L chain V region of human antibody 12B5 binding to human MPL was designed by connecting the nucleotide sequence of gene (SEQ ID NO: 67) at the 5'-end to the leader sequence (SEQ ID NO: 68) originated from human antibody gene 3D6 (Nuc. Acid Res. 1990: 18; 4927). In the 25 same way as mentioned above the designed nucleotide sequence was divided into four oligonucleotides having overlapping

sequences of 15 bp each (12B5VL-1, 12B5VL-2, 12B5VL-3, 12B5VL-4) and synthesized respectively. 12B5VL-1 (SEQ ID NO: 69) and 12B5VL-3 (SEQ ID NO: 71) had sense sequences, and 12B5VL-2 (SEQ ID NO: 70) and 12B5VL-4 (SEQ ID NO: 72) had antisense sequences, respectively. Each of the synthesized oligonucleotides was assembled by respective complementarity and mixed with the external primer (12B5VL-S and 12B5VL-A) to amplify the full length of the gene. 12B5VL-S (SEQ ID NO: 73) was designed to hybridize to 5'-end 10 of the leader sequence by the forward primer and to have Hind III restriction enzyme recognition site and Kozak sequence. 12B5VL-A (SEQ ID NO: 74) was designed to hybridize to the nucleotide sequence encoding C-terminal of L chain V region by the reverse primer and to have a splice 15 donor sequence and BamHI restriction enzyme recognition site.

Performing the PCR as mentioned above, the PCR product was purified by 1.5% low-melting-temperature agarose gel (Sigma), digested by restriction enzymes BamHI and Hind III, and cloned into an expression vector HEF-g $\kappa$  for human L chain. After determining the DNA sequence the plasmid containing the correct DNA sequence was named HEF-12B5L-g $\kappa$ . The nucleotide sequence and amino acid sequence of the reconstructed 12B5 L chain V region which were included in plasmid HEF-12B5L-g $\kappa$  are shown in SEQ ID NO:75.

25 7.3 Production of reconstructed 12B5 single chain Fv (scFv)

The reconstructed 12B5 antibody single chain Fv was designed to be in the order of 12B5VH-linker-12B5VL and to

have FLAG sequence (SEQ ID NO: 76) at C-terminal to facilitate the detection and purification. The reconstructed 12B5 single chain Fv (sc12B5) was constructed using a linker sequence consisting of 15 amino acids represented by (Gly<sub>4</sub>Ser)<sub>3</sub>.

5 (1) Production of the reconstructed 12B5 single chain Fv using the linker sequence consisting of 15 amino acids

The gene encoding the reconstructed 12B5 antibody single chain Fv, which contained the linker sequence consisting of 15 amino acids, was constructed by connecting 12B5 H chain V region, linker region and 12B5 L chain V region which was amplified by PCR respectively. This method is schematically shown in Fig. 47. Six PCR primers (A-F) were used for production of the reconstructed 12B5 single chain Fv. Primers A, C, and E had sense sequences, and primers B, D, and F had antisense sequences.

10 The forward primer 12B5-S (Primer A, SEQ ID NO: 77) for H chain V region was designed to hybridize to 5'-end of H chain leader sequence and to have EcoRI restriction enzyme 15 recognition site. The reverse primer HuVHJ3 (Primer B, SEQ ID NO: 78) for H chain V region was designed to hybridize to DNA encoding C-terminal of H chain V region.

20 The forward primer RHUJH3 (Primer C, SEQ ID NO: 79) for the linker was designed to hybridize to DNA encoding the N-terminal of the linker and to overlap DNA encoding the C-terminal of H chain V region. The reverse primer RHuVK1 25 (Primer D, SEQ ID NO: 80) for the linker was designed to

hybridize to DNA encoding the C-terminal of the linker and overlap DNA encoding the N-terminal of L chain V region.

The forward primer HuVK1.2 (Primer E, SEQ ID NO: 81) for L chain V region was designed to hybridize to DNA encoding the N-terminal of L chain V region. The reverse primer 12B5F-A for L chain V region (Primer F, SEQ ID NO: 82) was designed to hybridize to DNA encoding C-terminal of L chain V region and to have the sequence encoding FLAG peptide (Hopp, T. P. et al., Bio/Technology, 6, 1204-1210, 1988), two transcription stop codons and NotI restriction enzyme recognition site.

In the first PCR step, three reactions A-B, C-D, and E-F were performed, and the three PCR products obtained from the first step PCR were assembled by respective complementarity. After adding primers A and F the full length DNA encoding the reconstructed 12B5 single chain Fv having the linker consisting of 15 amino acids was amplified (the second PCR). In the first step PCR, the plasmid HEF-12B5H-g $\gamma$ 1 (see Example 7. 1) encoding the reconstructed 12B5 H chain V region, pSCFVT7-hM21 (humanized ONS-M21 antibody) (Ohtomo et al., Anticancer Res. 18 (1998), 4311-4316) containing DNA (SEQ ID NO: 83) encoding the linker region consisting of Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser (Huston et al., Proc. Natl. Acad. Sci. USA, 85, 5879-5883, 1988) and the plasmid HEF-12B5L-g $\kappa$  (see Example 7. 2) encoding the reconstructed 12B5 L chain V region were used as templates, respectively.

50 $\mu$ l of PCR solution for the first step contained 5 $\mu$ l of 10 x PCR Gold Buffer II, 1.5mM MgCl<sub>2</sub>, 0.08mM dNTPs, 5 units of DNA polymerase AmpliTaq Gold (all by PERKIN ELMER), each 100 pmole of each primer and 100ng of each template DNA.

5 The PCR solution was heated at 94°C of the initial temperature for 9 minutes, at 94 for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. After repeating the cycle 35 times the reaction mixture was further heated 72°C for 5 minutes.

10 The PCR products A-B, C-D, and E-F were assembled by the second PCR. PCR mixture solution for the second step of 98 $\mu$ l containing as the template 1 $\mu$ l of the first PCR product A-B, 0.5 $\mu$ l of PCR product C-D and 1 $\mu$ l of PCR product E-F, 10 $\mu$ l of 10 x PCR Gold Buffer II, 1.5mM MgCl<sub>2</sub>, 0.08mM dNTPs, 15 5 units of DNA polymerase AmpliTaq Gold (all by PERKIN ELMER) was heated at 94°C of the initial temperature for 9 minutes, at 94°C for 2 minutes, at 65°C for 2 minutes and 72°C for 2 minutes. After repeating the cycle two times, each 100 pmole of each of primers A and F were added. After 20 repeating the cycle consisting of at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute 35 times, the reaction mixture was heated at 72°C for 5 minutes.

25 The DNA fragments produced by the second PCR were purified using 1.5% low-melting-temperature agarose gel, digested by EcoRI and NotI, and cloned into pCHO1 vector and pCOS1 vector (Japanese Patent Application No. 8-255196). The expression vector pCHO1 was a vector constructed by

deleting the antibody gene from DHFR- $\Delta$ E-rvH-PM1-f (see WO92/19759) by EcoRI and SmaI digestion, and connecting to EcoRI-NotI-BamHI Adaptor (TAKARA SHUZO). After determining the DNA sequence the plasmids containing the DNA fragment encoding the correct amino acid sequence of reconstructed 12B5 single chain Fv were named pCHO-sc12B5 and pCOS-sc12B5. The nucleotide sequence and amino acid sequence of the reconstructed 12B5 single chain Fv included in the plasmids pCHO-sc12B5 and pCOS-sc12B5 are shown in SEQ ID NO: 84.

10 7.4 Expression of antibody 12B5 (IgG, Fab) and single chain Fv polypeptide by animal cell

Antibody 12B5 (IgG, Fab) and single chain Fv derived from antibody 12B5 were expressed by using COS-7 cells or CHO cells.

15 The transient expression using COS-7 cells was performed as follows. The transfection was performed by electroporation method using Gene Pulser equipment (BioRad). For the expression of antibody 12B5 (IgG) each 10 $\mu$ g of the above-mentioned expression vector HEF-12B5H-g $\gamma$ 1 and HEF-12B5L-g $\kappa$  were added, for the expression of 12B5Fab fragment each 10 $\mu$ g of pFd-12B5H and HEF-12B5L-g $\kappa$  were added and for the expression of single chain Fv 10 $\mu$ g of pCOS-sc12B5 was added to COS-7 cells ( $1 \times 10^7$  cells/ml) suspended in 0.8ml of PBS. The mixture kept in a cuvette was treated by pulse at the capacity of 1.5kV, 25 $\mu$ FD. After recovering for 10 minutes in a room temperature the electroporated cells were added to DMEM culture medium (GIBCO BRL) containing 10%

bovine fetal serum cultivated. After cultivating overnight the cells were washed once by PBS, added to serum-free medium CHO-S-SFM II and cultivated for 2 days. The culture medium was centrifuged to remove cell debris and filtered 5 with 0.22 $\mu$ m filter to prepare the culture supernatant.

To establish a stable expression CHO cell line for the single chain Fv (polypeptide) derived from antibody 12B5, the expression vector pCHO-sc12B5 was introduced into CHO cells as follows.

10 The expression vector was introduced into CHO cells by electroporation method using Gene Pulser equipment (BioRad). Linearized DNA (100 $\mu$ g) obtained by digestion with restriction enzyme PvulI and CHO cells ( $1 \times 10^7$  cells /ml) suspended in 0.8 ml of PBS were mixed in a cuvette, left 15 stationary on ice for 10 minutes and treated with pulse at the capacity of 1.5kV, 25 $\mu$ FD. After recovering for 10 minutes at a room temperature the electroporated cells were added to CHO-S-SFM II (GIBCO BRL) containing 10% bovine fetal serum and cultivated. After cultivating for 2 days 20 the cultivation was continued in CHO-S-SFM II (GIBCO BRL) containing 5nM methotrexate (SIGMA) and 10% bovine fetal serum. From thus obtained clones a clone with high expression rate was selected as the production cell line for 25 12B5 single chain Fv. After cultivating in serum-free medium CHO-S-SFM II (GIBCO BRL) containing 10nM methotrexate (SIGMA), the culture supernatant was obtained by centrifugal separation of cell debris.

7.5 Purification of single chain Fv derived from 12B5 produced by CHO cells

The culture supernatant of CHO cell line expressing 12B5 single chain Fv obtained in 7.4 was purified by anti-FLAG antibody column and gel filtration column.

5 (1) Anti-FLAG antibody column

The culture supernatant was added to anti-FLAG M2 affinity gel (SIGMA) equilibrated by PBS. After washing the column by the same buffer the proteins adsorbed to the column were 10 eluted by 0.1M glycine-HCl buffer (pH 3.5). The eluted fractions were immediately neutralized by adding 1M Tris-HCl buffer (pH 8.0). The eluted fractions were analyzed by SDS-PAGE and the fraction which was confirmed to contain the single chain Fv was concentrated using Centricon-10 15 (MILLIPORE).

(2) Gel filtration

The concentrated solution obtained in (1) was added to Superdex200 column (10x300mm, AMERSHAM PHARMACIA) equilibrated by PBS containing 0.01% Tween20.

20 The product sc12B5 was eluted in two peaks (A, B) (see Fig. 48). The fractions A and B were analyzed using the 14%-SDS-polyacrylamide gel. The sample was processed by electrophoresis in the presence and absence of a reducing agent according to Laemmli method, and stained by Coomassie 25 Brilliant Blue after the electrophoresis. As shown in Fig. 49 the fractions A and B, regardless of the presence of the reducing agent or its absence, produced a single band having

an apparent molecular weight of about 31 kD. When the fractions A and B were analyzed by gel filtration using Superdex200 PC 3.2/30 (3.2x300mm, AMERSHAM PHARMACIA), the fraction A produced an eluted product at an apparent molecular weight of about 44 kD and the fraction B produced at 22kD (see Fig. 50a and b). The results show that the fraction A is the non-covalent bond dimer of sc12B5 single chain Fv, and B is the monomer.

7.6 Measurement of TPO-like agonist activity of various 10 single chain Fvs

The TPO-like activity of anti-MPL single chain antibody was evaluated by measuring the proliferation activity to Ba/F3 cells (BaF/mpl) expressing human TPO receptor (MPL). After washing BaF/Mpl cells two times by 15 RPMI1640 culture medium (GIBCO) containing 10% bovine fetal serum (HyClone), the cells were suspended in the culture medium at cell density of  $5 \times 10^5$  cells/ml. The anti-MPL single chain antibody and human TPO (R&D Systems) was diluted with the culture medium, respectively. 50 $\mu$ l of the 20 cell suspension and 50 $\mu$ l of the diluted antibody or human TPO were added in 96-well microplate (flat bottom) (Falcon), and cultivated in CO<sub>2</sub> incubator (CO<sub>2</sub> concentration: 5%) for 24 hours. After the incubation 10 $\mu$ l of WST-8 reagent (reagent for measuring the number of raw cells SF: Nacalai 25 Tesque) was added and the absorbance was immediately measured at measurement wavelength of 450nm and at refference wavelength of 620nm using fluorescence absorbency

photometer SPECTRA Fluor (TECAN). After incubating in CO<sub>2</sub> incubator (CO<sub>2</sub> concentration: 5%) for 2 hours, the absorbance at 450nm of measurement wavelength and 620nm of refference wavelength was again measured using SPECTRA Fluor.

5 Since WST-8 reagent developed the color reaction depending upon the number of live cells at wavelength of 450nm, the proliferation activity of BaF/Mpl was evaluated based on the change of absorbance in 2 hours.

10 The results of the agonist activity to MPL measured by using culture supernatants of COS-7 cells expressing various 12B5 antibody molecules showed as illustrated in Fig. 51 that

15 12B5IgG having bivalent antigen-binding site increased the absorbance in concentration-dependent manner and had TPO-like agonist activity (ED50; 29nM), while the agonist

activity of 12B5Fab having monovalent antigen-bidind site was very weak (ED50; 34,724nM). On the contrary the single chain Fv (sc12B5) having monovalent antigen-binding site like Fab showed strong agonist activity at a level that ED50

20 was 75nM. However it had been known that variable regions of H chain and L chain of the single chain Fv were associated through non-covalent bond and, therefore, each variable region was dissociated in a solution and could be associated with variable region of other molecule to form multimers like dimers. When the molecular weight of sc12B5

25 purified by gel filtration was measured, it was confirmed that that there were molecules recognized to be monomer and dimer (see Fig. 48). Then monomer sc12B5 and dimer sc12B5

were isolated (see Fig. 50) and measured for the agonist activity to MPL. As shown in Figs. 51 and 52, ED50 of sc12B5 monomer was 4438.7nM, which confirmed that the agonist activity was reduced compared with the result using culture supernatant of COS-7 cells. On the contrary single chain Fv (sc12B5 dimer) having bivalent antigen-binding site showed about 400-fold stronger agonist activity (ED50; 10.1nM) compared with monovalent sc12B5. Furthermore, the bivalent single chain Fv showed the agonist activity equivalent to or higher than the agonist activity of human TPO and 12B5IgG.

**EXPLANATION OF DRAWINGS**

Figure 1 shows the result of flow cytometry, illustrating that human IgG antibody does not bind to L1210 cells expressing human IAP (hIAP/L1210).

Figure 2 shows the result of flow cytometry, illustrating that the chimera MABL-1 antibody specifically binds to L1210 cells expressing human IAP (hIAP/L1210).

Figure 3 shows the result of flow cytometry, illustrating that the chimera MABL-2 antibody specifically binds to L1210 cells expressing human IAP (hIAP/L1210).

Figure 4 schematically illustrates the process for producing the single chain Fv according to the present invention.

Figure 5 illustrates a structure of an expression plasmid which can be used to express a DNA encoding the single chain Fv of the invention in E. coli.

Figure 6 illustrates a structure of an expression plasmid which is used to express a DNA encoding the single chain Fv of the invention in mammalian cells.

Figure 7 shows a photograph showing the result of western blotting in Example 5.4. From the left, a molecular weight marker (which indicates 97.4, 66, 45, 31, 21.5 and 14.5 kDa from the top), the culture supernatant of pCHO1-introduced COS7 cells and the culture supernatant of pCHOM2-introduced COS7 cells. It illustrates that the reconstructed single chain Fv of the antibody MABL-2 (arrow) is contained in the culture supernatant of the pCHOM2-introduced cells.

Figure 8 shows the result of flow cytometry, illustrating that an antibody in the culture supernatant of pCHO1/COS7 cell as a control does not bind to pCOS1/L1210 cell as a control.

Figure 9 shows the result of flow cytometry, illustrating that an antibody in the culture supernatant of MABL2-scFv/COS7 cells does not bind to pCOS1/L1210 cells as a control.

Figure 10 shows the result of flow cytometry, illustrating that an antibody in the culture supernatant of pCOS1/COS7 cells as a control does not bind to hIAP/L1210 cells.

Figure 11 shows the result of flow cytometry, illustrating that an antibody in the culture supernatant of MABL2-scFv/COS7 cells specifically binds to hIAP/L1210 cells.

5       Figure 12 shows the result of the competitive ELISA in Example 5.6, wherein the binding activity of the single chain Fv of the invention (MABL2-scFv) to the antigen is demonstrated in terms of the inhibition of binding of the mouse monoclonal antibody MABL-2 to the antigen as an index, in comparison with the culture supernatant of pCHO1/COS7 cells as a control.

10      Figure 13 shows the results of the apoptosis-inducing effect in Example 5.7, illustrating that the antibody in the culture supernatant of pCHO1/COS7 cells as a control does not induce the apoptosis of pCOS1/L1210 cells as a control.

15      Figure 14 shows the results of the apoptosis-inducing effect in Example 5.7, illustrating that the antibody in the culture supernatant of MABL2-scFv/COS7 cells does not induce apoptosis of pCOS1/L1210 cells as a control.

20      Figure 15 shows the results of the apoptosis-inducing effect in Example 5.7, illustrating that the antibody in the culture supernatant of pCHO1/COS7 cells as a control does not induce apoptosis of hIAP/L1210 cells.

Figure 16 shows the results of the apoptosis-inducing effect in Example 5.7, illustrating that the antibody in the culture supernatant of MABL2-scFv/COS7 cells specifically induces apoptosis of hIAP/L1210 cells.

25      Figure 17 shows the results of the apoptosis-inducing effect in Example 5.7, illustrating that the antibody in the culture supernatant of pCHO1/COS7 cells as a

control does not induce apoptosis of CCRF-CEM cells (at 50% of the final concentration).

Figure 18 shows the results of the apoptosis-inducing effect in Example 5.7, illustrating that the antibody in the culture supernatant of MABL2-scFv/COS7 cells specifically induces apoptosis of CCRF-CEM cells (at 50% of the final concentration).

Figure 19 shows the chromatogram obtained in the purification of the single chain Fv derived from the antibody MABL-2 produced by the CHO cells in Example 5.9, illustrating that fraction A and fraction B were obtained as the major peaks when the fraction from Blue-sepharose column was purified with hydroxyapatite column.

Figure 20 shows the results of purification by gel filtration of fraction A and fraction B obtained in Example 5.9-(2), illustrating that the major peaks (AI and BI, respectively) were eluted from fraction A at approximately 36 kD of the apparent molecular weight and from fraction B at approximately 76 kD.

Figure 21 is the analysis on SDS-PAGE of the fractions obtained in the purification of the single chain Fv derived from the antibody MABL-2 produced by the CHO cells in Example 5.9, illustrating that a single band of approximately 35 kD of molecular weight was observed in both fractions.

Figure 22 shows the results of analysis of fractions AI and BI obtained by gel filtration in the

purification of the single chain Fv derived from the antibody MABL-2 produced by the CHO cells, wherein fraction AI comprises monomer and fraction BI comprises dimer.

5 Figure 23 illustrates a structure of an expression plasmid which can be used to express a DNA encoding the single chain Fv of the invention in E. coli.

10 Figure 24 shows the results of purification on the gel filtration column of crude products of the single chain Fv polypeptide derived from the antibody MABL-2 produced by E. coli obtained in Example 5.12, wherein each peak indicates monomer or dimer, respectively, of the single chain Fv produced by E. coli.

15 Figure 25 shows the results of the apoptosis-inducing effect in Example 5.13, illustrating that mouse IgG antibody as a control does not induce apoptosis of hIAP/L1210 cells (the final concentration of 3  $\mu$ g/ml).

20 Figure 26 shows the results of the apoptosis-inducing effect in Example 5.13, illustrating that the dimer of MABL2-scFv produced by the CHO cells remarkably induces apoptosis of hIAP/L1210 cells (the final concentration of 3  $\mu$ g/ml).

25 Figure 27 shows the results of the apoptosis-inducing effect in Example 5.13, illustrating that the dimer of MABL2-scFv produced by E. coli remarkably induces apoptosis of hIAP/L1210 cells (the final concentration of 3  $\mu$ g/ml).

Figure 28 shows the results of the apoptosis-inducing effect in Example 5.13, illustrating that apoptosis induction to hIAP/L1210 cells by the MABL2-scFv monomer produced by the CHO cells is the same level as that of the control (the final concentration of 3  $\mu$ g/ml).

Figure 29 shows the results of the apoptosis-inducing effect in Example 5.13, illustrating that apoptosis induction to hIAP/L1210 cells of the MABL2-scFv monomer produced by E. coli is the same level as that of control (the final concentration of 3  $\mu$ g/ml).

Figure 30 shows the results of the apoptosis-inducing effect in Example 5.13, illustrating that mouse IgG antibody used as a control does not induce apoptosis of hIAP/L1210 cells even when anti-FLAG antibody is added (the final concentration of 3  $\mu$ g/ml).

Figure 31 shows the results of the apoptosis-inducing effect in Example 5.13, illustrating that MABL2-scFv monomer produced by the CHO cells remarkably induces apoptosis of hIAP/L1210 cells when anti-FLAG antibody is added (the final concentration of 3  $\mu$ g/ml).

Figure 32 shows the results of quantitative measurement of human IgG in the serum of a human myeloma cell line KPMM2-transplanted mouse, indicating amounts of human IgG produced by the human myeloma cells in the mouse. It illustrates that the dimer of scFv/CHO remarkably inhibited growth of the KPMM2 cells.

Figure 33 shows the survival time of the mouse after the transplantation of tumor, illustrating that the scFv/CHO dimer-administered group elongated remarkably the survival time.

5 Figure 34 illustrates a structure of an expression plasmid which expresses a modified antibody [sc(Fv)<sub>2</sub>] comprising two H chain V regions and two L chain V regions derived from the antibody MABL-2.

10 Figure 35 illustrates a structure of a plasmid which expresses a scFv (HL type) wherein the V regions are linked in the manner of [H chain]-[L chain] without a peptide linker.

Figure 36 illustrates a structure of the HL-type polypeptide and amino acid sequences of peptide linkers.

15 Figure 37 illustrates a structure of a plasmid which expresses a scFv (LH type) wherein the V regions are linked in the manner of [L chain]-[H chain] without a peptide linker.

20 Figure 38 illustrates a structure of the LH-type polypeptide and amino acid sequences of peptide linkers.

Figure 39 shows the results of the western blotting in Example 6.4, illustrating that the modified antibody sc(FV)<sub>2</sub> comprising two H chain V regions and two L chain V regions, and the MABL2-scFv having peptide linkers 25 with different length are expressed.

Figures 40a and 40b show the results of flow cytometry using the culture supernatant of COS7 cells

prepared in Example 6.3 (1), illustrating that the MABL2-scFv and sc(Fv)<sub>2</sub> having peptide linkers with different length have high affinities against human IAP.

5 Figure 41 shows the results of the apoptosis-inducing effect in Example 6.6, illustrating that the scFv <HL3, 4, 6, 7, LH3, 4, 6 and 7> and the sc(Fv)<sub>2</sub> remarkably induce cell death of hIAP/L1210 cells.

10 Figure 42 shows the results of the evaluation of antigen binding capacity in Example 6.10, illustrating that the dimer of scFv <HL5> and sc(Fv)<sub>2</sub> have high affinities against human IAP.

15 Figure 43 shows the results of the in vitro apoptosis-inducing effect in Example 6.11, illustrating that the dimer of scFv <HL5> and the sc(Fv)<sub>2</sub> induce apoptosis of hIAP/L1210 cells and CCRF-CEM cells in concentration-dependent manner.

20 Figure 44 shows the results of the quantitative measurement of M protein produced by a human myeloma cell line KPMM2 in the serum of the human myeloma cell-transplanted mouse. It illustrates that the dimer of scFv <HL5> and the sc(Fv)<sub>2</sub> remarkably inhibited growth of the KPMM2 cells.

25 Figure 45 shows the survival time (days) of mice after the transplantation of tumor, illustrating that the survival time of the scFv <HL5> administrated-group was remarkably prolonged.

Figure 46 shows the survival time (days) of mice after the transplantation of tumor, illustrating that the survival time of the sc(Fv)<sub>2</sub> administrated-group was remarkably prolonged.

5           Figure 47 is a scheme showing the method for constructing DNA fragment encoding the reconstructed 12B5 single chain Fv containing the linker sequence consisting of 15 amino acids and the structure thereof.

10           Figure 48 shows the purification result of each 12B5 single chain Fv by gel filtration obtained in Example 7. 5 (1), illustrating that sc12B5 was divided into two peaks (fractions A and B).

15           Figure 49 shows the analytical result of each fraction A and B by SDS-PAGE performed in Example 7. 5 (2).

20           Figure 50 shows the analytical result of each fraction A and B by Superdex200 column performed in Example 7. 5 (2), illustrating that the major peak of fraction A was eluted at an apparent molecular weight of about 44 kD shown in (a) and that the major peak of fraction B was eluted at an apparent molecular weight of about 22kD shown in (b).

25           Figure 51 shows the measurement result of the TPO-like agonist activity of sc12B5 and antibody 12B5 (IgG, Fab), illustrating that 12B5IgG and monovalent single chain Fv (sc12B5) showed TPO-like agonist activity in concentration-dependent manner.

Figure 52 shows the measurement result of TOP-like agonist activity of sc12B5 monomer and dimer, illustrating

that single chain Fv (sc12B5 dimer) having bivalent antigen-binding site had agonist activity about 400-fold higher than monovalent sc12B5 and that the efficacy is equivalent to or higher than human TPO.

5

#### **INDUSTRIAL APPLICABILITY**

The modified antibodies of the invention have an agonist action capable of transducing a signal into cells by crosslinking a cell surface molecule(s) and are advantageous in that the permeability to tissues and tumors is high due to the lowered molecular size compared with antibody molecule (whole IgG). The modified antibodies have remarkably higher activity compared with the original antibodies, which is attributable to that the modified antibodies are in a shape closer to a ligand compared with original antibodies. Therefore the modified antibodies can be used as signal-transducing agonists. The modification of antibody molecule results in the reduction of side effects caused by intercellular crosslinking and provides novel medicines inducing only required action by crosslinking a cell surface molecule(s). Medical preparations containing as active ingredient the modified antibody of the invention are useful as preventives and/or remedies for cancers, inflammation, hormone disorders and blood diseases, for example, leukemia, malignant lymphoma, aplastic anemia, myelodysplasia syndrome and polycythemia vera.